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Study of shanny (*Lipophrys pholis*) life cycle inferred from microestructure and microchemistry of otoliths: ontogeny, coastal recruitment and connectivity

Ana Margarida Gama Carvalho

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Tese de Doutoramento apresentada à
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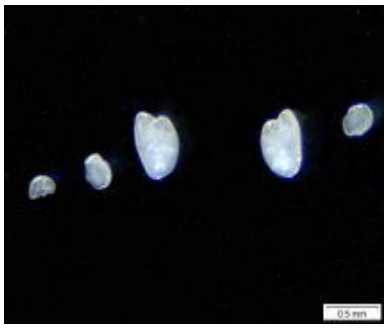

Study of shanny (*Lipophrys pholis*) life cycle inferred from microstructure and microchemistry of otoliths: ontogeny, coastal recruitment and connectivity

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Doutoramento em Biologia
Biologia
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Foreword

According to the number 7 of Article 6 from the Regulation of the Doctoral Program in Biology, Faculdade de Ciências da Universidade do Porto (and in agreement with the Portuguese Law Decree nº 74/2006), this thesis integrates the articles and poster/oral comunications listed below, written in collaboration with co-authors. The candidate hereby declares that she contributed to conceiving the ideas, compiling and producing the data and analyzing the data, and also declares that she led the writing of all Chapters.

Articles in indexed journals

- Carvalho MG, Moreira C, Albuquerque R, Daros FA, Swearer SE, Queiroga H, Santos PT, Correia AT (In preparation) Use of otolith natal elemental signatures as natural tags to evaluate the larval dispersion, coastal recruitment, habitat connectivity and population structure of *Lipophrys pholis*

- Carvalho MG, Moreira C, Cardoso JFMF, Brummer GVP, Veer HW, Queiroga H, Santos PT, Correia AT. 2017. (In press). Movement and connectivity in the fish *Lipophrys pholis* (Linnaeus, 1758) revealed by otolith oxygen and carbon stable isotopes. Marine Biology Research. http://dx.doi.org/10.1080/17451000.2017.1306079

- Carvalho MG, Moreira C, Queiroga H, Santos PT, Correia AT. 2017. Age, growth and sex of the shanny, *Lipophrys pholis* (Linnaeus, 1758) (Teleoste Blenniidae), from the NW coast of Portugal. Journal of Applied Ichthyology. 33:242-251. http://dx.doi.org/10.1111/jai.13307

- Carvalho MG, Moreira AS, Moreira C, Queiroga H, Santos PT, Correia AT. 2017. Pelagic larval duration, size at settlement and coastal recruitment of shanny *Lipophrys pholis* as revealed from otolith microstructure. Journal of the Marine Biological Association of the United Kingdom. 97 (1): 197-205 http://dx.doi.org/10.1017/S0025315415002179

- Carvalho MG, Moreira C, Queiroga H, Santos PT, Correia AT. 2015. Ontogenetic development of the sagittal otoliths of *Lipophrys pholis* during the embryonic, larval and settlement stages. Ichthyological Research 62 (3): 351-356.

- Carvalho MG, Moreira AS, Moreira C, Queiroga H, Santos PT, Correia AT. 2014. Validation of otolith daily increments in early juveniles of shanny *Lipophrys pholis*. Journal of Fish Biology 84: 1234-1239. http://dx.doi.org/10.1111/jfb.12333

Poster and oral communications

- Carvalho MG, Moreira AS, Moreira C, Queiroga H, Santos PT, Correia AT. 2015. New insights into early history traits of *Lipophrys pholis* inferred from otolith microstructure studies. XV European Congress of Ichthyology. 7-11 september. Oporto, Portugal. Oral presentation.

- Carvalho MG, Moreira AS, Moreira C, Queiroga H, Santos PT, Correia AT. 2014. Validation in otolith daily increments in early juveniles of *Lipophrys pholis.* IJUP 2014. february. Oporto,Portugal. Poster.

- Carvalho MG, Moreira C, Queiroga H, Santos PT, Correia AT. 2014. Ontogenetic development of the sagittal otoliths of *Lipophrys pholis* during the embryonic, larval and settlement stages. Jornadas Ibéricas de Ictiologia. 24-27 june. Lisbon, Portugal. Poster.

- Carvalho MG, Moreira AS, Moreira C, Queiroga H, Santos PT, Correia AT. 2014. Pelagic larval duration, size at settlement and coastal recruitment of shanny *Lipophrys pholis* as revealed from otolith microstructure. ICBF2014. 3-7 august. Edinburgh, Scotland. Oral presentation.

- Carvalho MG, Moreira C, Rodrigues S, Queiroga H, Santos PT, Correia AT. 2014.Age, growth and sex of *Lipophrys pholis* from the North coast of Portugal. SIEBM 2014. 2-5 september. Gijon, Spain. Poster.

- Carvalho MG, Moreira C, Cardoso J, Brumme Geert-Jan, Gaever PV, Queiroga H, Santos PT, Correia AT.2014. Use of stable isotopes (oxygen and carbon) in otoliths to study fish movement and connectivity in *Lipophrys pholis*. IOS2014. 20-24 october. Mallorca. Spain.Oral presentation.

- Moreira AS, Carvalho MG, Moreira M, Daros FA, Queiroga H, Santos PT, Correia AT. 2013. Study of the coastal recruitment of *Lipophrys pholis* in the Portuguese occidental coast inferred from otolith microstructure analysis. XV Congresso Latino Americano de ciências do mar. 27-31 october. Punta del Este, Uruguai. Poster

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Abstract

The ontogenetic development of the sagittal otoliths of *Lipophrys pholis* during embryonic, larval, settlement and recruitment stages was examined from eggs, new settlers and recruits collected during low tides in several Portuguese rocky beaches. The eggs were reared under controlled laboratory conditions until the larval stage. Viewed by scanning electron microscopy, sagittae recorded eight to ten micro-increments before hatching in late embryonic phases. In larvar otoliths a visible hatching check was observed and micro-increments were deposited on a daily basis. Early settlers presented in the otolith edge two types of settlement marks (Ia and Ib). Furthermore micro-increments in sagittae were also shown to be deposited daily in early juveniles using fluorescent dyes. Pelagic larval duration estimated from micro-increment counts until the settlement marks in recruits collected along the occidental Portuguese coast ranged from 57 to 73 days. Moreover, pelagic larval duration showed a latitudinal reduction trend from North to South, probably related with the regional seawater temperatures. Settlement sizes did not show, however, any regional differences suggesting to be a more conservative character within the species. Age at coastal recruitment varied between 85 and 109 days, but northern individuals were recruited with an older age. Back - calculated spawning, hatching and settlement dates appear to be unrelated to the lunar cycle for *L. pholis*. Age, growth, sex and gonadal maturation of *L. pholis* collected seasonally in a northern Portuguese rocky beach (Póvoa do Varzim) throughout one year were evaluated. Marginal increments analysis showed that one translucent and one opaque zone were formed each year in the sagittal otoliths. Age for the *L. pholis* ranged from 0 to 6 years. Maximum gonadosomatic index for males and females corresponded with the breeding season (November and March). The estimated Length and age at first maturity were recorded in 70 mm and before one year old. Otolith oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) isotopic signatures were determined by isotope-ratio mass spectrometry to assess the degree of separation between individuals collected across Portuguese coast. A total of one hundred and twenty individuals were collected in six rocky beaches (Agudela, Cabo do Mundo, Boa Nova – three sites within one region; Peniche, Sines and Olhos de Água – three additional regions). Mean otolith isotopic ratios obtained ranged from - 7.77‰ to -6.62‰ for $\delta^{13}C$ and -0.02‰ to 1.14‰ for $\delta^{18}O$. Both seawater temperature and salinity caused differences in otolith $\delta^{18}O$ among the four main sampling regions. The inter-regional variation in $\delta^{13}C$ was most likely related to slight differences in the diet. This study showed that otolith stable isotopic differences found at large spatial scales, at least for the occidental northern and central regions, indicate that *L. pholis* experience different environmental conditions during their juvenile phase and/or limited movements between regions. Stable isotopic ratios also suggest high connectivity and/or a

larval retention at small spatial scales. Otolith's geochemical signatures can be used as a tool to identify the fish source origin once they have the capacity to reflect the water chemistry of the environment here fish lived. *L. pholis* embryos and recruits from the same cohort were collected in 2013 from 17 sites within 3 main regions of the Portuguese coast (NW, CW and S regions). Laser ablation inductively coupled plasma mass spectrometry was used to measure the concentration of 7 informative elemental ratios in the otolith's core. Molar ratios of Li/Ca, Mg/Ca, P/Ca, S/Ca, Mn/Ca, Sr/Ca and Ba/Ca show that natal chemical signatures are somewhat spatially specific for *L. pholis*. However multi-elemental signatures are highly different between sub-regions and/or sites. The population connectivity matrix identified diferent dispersal pathways for *L. pholis* embryos. Cabo do Mundo was an important source population for the NW region. For the CW region, Peniche was the site that most contributed as source population for Estremadura Norte sub region; Praia das Maças was the site that contribute most for Estemadura Sul population; and Alpertuche was the greatest contribution for Arrábida sub region source. SW registered similar values for self-recruitment for both Odeceixe and Sines sites. 20% to 53 % of early juveniles may be returning to their natal population (self-recruitment), but others came from other areas mainly from southern locations. This also means that fish larvae disperse away from their natal population so that local populations operate as 'open' systems driven by recruitment of larvae from other sub-populations suggests a metapopulation structure. Furthermore, unexpectedly, the SW is the main contributor for all the main regions and larvae are probably driven by the northward flow of the Portuguese Coastal Counter Current during winter suggesting that long-distance dispersal is the norm for *L. pholis* fish populations. However this data should be looked with care and further studies should assess if this pattern persists in subsequents years.

Keywords: Bleniidae, sagittae, microstructure, microchemistry, life cycle

Resumo

O desenvolvimento ontogenético dos otólitos sagitta de *Lipophrys pholis,* foi estudado durante os estadios embrionário, larvar, assentamento e recrutamento através de ovos, recém-assentados e recrutas, recolhidos durante a maré baixa em algumas praias rochosas Portuguesas. Os ovos foram "criados" em condições controladas de laboratório até à fase larvar. Observações dos sagitta ao microscópico electrónico de varrimento, registaram 8-10 micro-incrementos antes da eclosão, numa fase embrionária tardia. Nos otólitos das larvas, foi visivelmente observada uma marca de eclosão e os micro-incrementos foram depositados numa base diária. Os recém-assentados apresentaram na borda do otólito dois tipos de marca de assentamento (Ia e Ib). Além disso, nos sagittae dos juvenis, os micro-incrementos com recurso a marcadores fluorescentes também apresentaram uma deposição diária. A duração pelágica larvar, nos recrutas capturados ao longo da costa ocidental portuguesa estimada na contagem dos micro-incrementos até à marca de assentamento, variou de 57 a 73 dias. Também, a duração da fase pelágica larvar mostrou uma tendência de redução latitudinal do Norte para o Sul, provavelmente relacionada com as temperaturas regionais da água do mar. Os tamanhos de assentamento, não mostraram no entanto, nenhuma diferença regional, sugerindo ser uma característica mais conservadora dentro da espécie. A idade de recrutamento costeiro variou entre 85 e 109 dias, mas os indivíduos do norte recrutaram com maior idade. O retro cálculo das datas de postura, de eclosão e assentamento parecem não estar relacionados com os ciclos lunares. Adicionalmente, para estudar a idade, o crescimento, o sexo e a maturação das gónadas de *L. pholis*, 251 indivíduos (115 fêmeas, 99 machos e 37 indeterminados; comprimento variando entre 30 mm e 172 mm) foram capturados em quatro campanhas de amostragem, de novembro de 2013 a agosto de 2014, na maré baixa das praias rochosas do norte de Portugal (Póvoa do Varzim), com o auxílio de camaroeiros. A análise dos incrementos marginais mostrou que uma zona translúcida e uma zona opaca se formam todos os anos nos otólitos sagitta. A idade de *L. pholis* variou entre 0 e 6 anos. O máximo índice gonadossomático para machos e fêmeas coincidiu com a época de reprodução (Novembro e Março) e o comprimento estimado e idade de primeira maturação foi de 70 mm e antes do 1º ano de vida. As assinaturas isotópicas de oxigénio ($\delta^{18}O$) e de carbono ($\delta^{13}C$) nos otólitos foram determinadas por espectrometria de massa de razão isotópica a fim de avaliar o grau de separação entre indivíduos recolhidos ao longo da costa portuguesa. Um total de cento e vinte indivíduos foram amostrados em seis praias rochosas (Agudela, Cabo do Mundo, Boa Nova - três locais dentro de uma região, Peniche, Sines e Olhos de Água - três regiões adicionais). As razões isotópicas médias obtidas nos otólitos variaram de -7.77 ‰ a -6.62 ‰ para $\delta^{13}C$ e de -0.02 ‰ a 1.14 ‰ para $\delta^{18}O$. Tanto a

temperatura da água do mar como a salinidade causaram diferenças no isótopo $\delta^{18}O$ do otólito entre as quatro principais regiões de amostragem. A variação inter-regional no $\delta^{13}C$ está provavelmente mais relacionada com pequenas diferenças na dieta. Este estudo mostrou que a grande escala foram encontradas diferenças nos isotópicas estáveis dos otolitos, pelo menos para as regiões do norte e centro ocidentais, indicando que *L. pholis* experimentou diferentes condições ambientais durante sua fase juvenil e / ou movimentos limitados entre regiões. A pequena escala espacial, as razões isotópicas estáveis sugerem alta conectividade e / ou retenção larvar. Embriões de *L. pholis* e recrutas da mesma coorte foram recolhidos no ano de 2013, em 17 locais dentro de 3 regiões principais da costa portuguesa (regiões NW, CW e SW). A espectrometria de massa por plasma acoplado indutivamente por ablação a laser foi usada para medir a concentração de 7 razões elementares informativas no núcleo do otólito. As proporções molares de Li / Ca, Mg / Ca, P / Ca, S / Ca, Mn / Ca, Sr / Ca e Ba / Ca mostram que as assinaturas químicas natais são espacialmente específicas para *L. pholis*. No entanto, as assinaturas multi-elementais são altamente diferentes entre sub-regiões e / ou sites. A matriz de conectividade da população identificou caminhos de dispersão diferentes para embriões de *L.pholis.* Cabo do Mundo foi uma importante fonte de população para a região NW. Para a região CW, Peniche foi o local que mais contribuiu como população-fonte para a sub-região Estremadura Norte; a Praia das Maças foi o local que mais contribuiu para a população da Estemadura Sul; e Alpertuche foi a maior contribuição para a sub-região da Arrábida. SW registou valores semelhantes para o auto-recrutamento para os locais de Odeceixe e Sines. 20% a 53% dos novos juvenis podem estar a regressar à sua população natal (auto-recrutamento), sendo que outros vêm de outras áreas, principalmente de locais do sul. Significa também que as larvas dispersam para fora da sua população natal e por isso as populações locais operam como sitemas “abertos” impulsionado pelo recrutamento de larvas de outras sub-populações sugerindo uma estrutura de metapopulação. Inesperadamente, o SW é o principal contribuinte para todas as principais regiões e, as larvas, são provavelmente impulsionadas pelo fluxo norte da Corrente Contígua Costeira Portuguesa durante o Inverno, sugerindo que a dispersão de longa distância é a norma para as populações de peixes de *L. pholis*. No entanto, estes dados devem ser analisados com cuidado e estudos adicionais devem avaliar se este padrão persiste em anos subsequentes.

Palavras- chave: Bleniidae, sagita, microestrutura, microquímica, ciclo de vida.

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Abbreviations and symbols

A: Anterior

ARS: Alizarin red S

Ca: Cortical alveoli

CR: corona radiata

CW: Central-West

D: Dorsal

GSI: Gonadossomatic Index

H_2O_2: Hydrogen Peroxide

HC: Hatching check

HCl: Hidrocloric acid

HSI: Hepatossomatic Index

I: Immature

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

II: Maturing or early oogenesis/spermatogenesis

III:Mature or mid oogenesis/spermatogenesis

IV:Spawning

IW: Increment Width

K: Condition Factor

KOH: Potassium hydroxide solution

L. pholis: *Lipophrys pholis*

LM: Light Microscopy

L_T: Total Length

n: nucleoli

N: nucleous

NW: North-West

O: Oocyte

OD: Otolith Diameter

OGR: Otolith Growth Rate

OR: Otolith Radius

P: Posterior

p: primordium

PGC: Primary germ cells,

PLD: Pelagic larval duration

R:Rostrum

SA: Sulcus Acusticus

SEM: Scanning Electron Microscopy

SM: Settlement Mark

Spc I: spermatocyte I

Spc II: spermatocyte II

Spg a: Spermatogonia type A

Spg b: spermatogonia type B

Spt: spermatids

Spz: spermatozoa

SSS: Seawater surface salinity

SST: Seawater surface temperature

SW: South-West

T: testis

TC: Tetracycline hidrocloride

Tg: testicular gland

TL: Total length

Total Age (A_{50}): Estimated total Age at first Maturity

Total Length (L_{50}): Estimated Total Length at first Maturity

UV: Ultraviolet

V: Spent

V: Ventral

VBGC: Von Bertalanffy growth curve

VPDP: Vienna Pee Dee Belemnite

VSMOW: Vienna Standard Mean Ocean Water

YG: Yolk platelets

YOY: Young of the year

ZG: zona granulosa

$\delta^{13}C$: Carbon isotope

$\delta^{18}O$: Oxygen isotope

CHAPTER 1

General Introdution

1.1. Framework and strategies

This work started in March 2013 and was part of the Larval Sources Project supported by the Portuguese Foundation for Science and Technology (European Regional Development Fund (ERDF) through the COMPETE – Operational Competitiveness Programme and by national funds through FCT – Foundation for Science and Technology, under the projects PEst-C/MAR/LA0015/2013 and PTDC/BIA-BIC/120483/ 2010.). The general scope of this project was to assess the ecological performance of marine protected area (MPA) networks using as models mussel *Mytilus galloprovinciali* and fish *Lipophys pholis.* The establishment and management of effective networks of MPAs is however hindered by the paucity of the data available for scientists and managers on fundamental aspects of the dynamics of marine populations. This paucity of data is basically rooted on the fact that most marine species have a bi-phasic life cycle, where a small planktonic larva precedes a benthic adult. Given that the small larvae are dispersed by marine currents in a medium with diffuse boundaries, local reproduction is decoupled from local recruitment and subpopulations of marine species are distributed over habitat patches that differ in quality, population growth rate and connectivity with the remaining subpopulations. Empirical and theoretical studies indicate that persistence of populations such as these depends on two basic mechanisms: i) self-persistence of local populations and ii) persistence that depends on connectivity among several local populations. The core objective of Larval Sources Project is to estimate connectivity between two Portuguese MPAs, the Reserva Natural das Berlengas and the Parque Natural da Arrábida, and among these and the remaining coast, using elemental fingerprinting of natal signatures deposited in *M. galloprovinciali* shells and in *L. pholis* larval otolith in order to obtain basic information to assess the factors that may influence their persistence and ecological performance.

However, the lack of scientific knowledge about the latter fish species, namely concerning its early life stage, led us to decide to focus this study to the *L. pholis* species. *Lipophrys pholis* is an abundant species in the Portuguese coastal area (rocky beaches) and easy to capture. This characteristic, in almost all life cycles stages, allowed us to perform sampling at various locations of the coast. The sampling design covered the entire distribution area of the species in the occidental Portuguese coast, during two consecutive years (2013 and 2014). An intensive fishing effort (one to two times per month) has been made around 20 sampling points, namely in Northwest Region (5 sites), Centre West Region (11 sites, including the two MPAs, the Reserva Natural das Berlengas and the Parque Natural da Arrábida) and Southwest region (4 sites). During this period in each campaign eggs/embryos, settlers/ recruits and adults were collected and, through the examination of otolith microstructure and

microchemistry some aspects of the early life history and connectivity of *Lipophrys pholis* was revealed. The study of the ontogenetic development of otoliths in *L. pholis* individuals during the embryonic, larval and juvenile/adult stages was made using Light microscopy and Scanning electronic microscopy. Hatching, first feeding, settlement, age at coastal recruitment and age at maturity events were tracked and validated. In recruits the validation of the daily grow rate of otoliths was assessed using fluorescent markers (tetracycline and alizarin). Additionally elemental analyses focused the different life-history stages across otolith transverse sections, namely in the core region of larvae/embryos and settlers/recruits. With recruits were established the use of stable isotope ratios (oxygen and carbon) in otoliths as ecological and environmental recorders of the fish habitat (namely, temperature and salinity) and as biological tracers for migration/connectivity.

1.2. Biology of *Lipophrys pholis*

Geographic distribution and habitat use

The shanny*, Lipophrys pholis* (Linnaeus, 1758), is one of the most abundant intertidal fishes in the NE Atlantic, extending its range from Mauritania to Norway, including the Azores and Madeira Islands, but also in the Mediterranean (Zander, 1986) (Fig. 1). It is also a common blenniid found in the Portuguese coastal areas, namely in the rocky beaches (Almada *et al.,* 2001).

Fig. 1- Distribution of *Lipophrys pholis* (www.fishbase.pt). The range colors indicate degree of suitable of habitat witch can be interpreted as possibility of occurrence (Red color- very probable to occur; yellow color– less probable to occur).

Blenniids are most abundant in the tropics, and most European species breed during spring and early summer (Zander, 1986). The majority of them either extends their ranges to West Africa and are especially abundant in the Mediterranean Sea. The geographic distribution of *L. pholis,* comparatively to other European blenniids, reaches much higher latitudes because it is probably a species adapted to cooler waters (Almada *et al.,* 1990a).

In the British Islands, *L. pholis* is of common occurrence in the lower half of the intertidal zone of rocky shores witch offer suitable shelter for this species. The relative abundance of the various size groups was observed, however, differed according to the water tidal levels. Smaller individuals, mainly 0 and 1 years old, were found farther up the shore, being more numerous in the region of midle-tide mark. They become less common at low water neap tide level and were found below it. Larger individuals, on the other hand, occurred low down on the shore, mainly at about the level of low water tides and sometimes slightly below (Qasim, 1957). In Portugal, a study of the pattern of spatial distribution and behavior of an intertidal fish assemblage on a rocky intertidal platform during high tides, showed that *L. pholis* is specifically abundant in the upper intertidal and were subject to larger displacement, up and down, with the tides (Faria & Almada, 2006).

Videotaping recorded the movements of *L. pholis* over almost an entire tidal cycle, showing that it was more active during the daytime. Also, there were more fish active in the morning and during the rising tide or in the high-tide period, than during the ebbing tide, presenting more convoluted paths during the rising tide (Burrows *et al.,* 1999). These authors suggested that the differences noted may be related to a foraging behavior. On the flood tide, at the beginning of the day and after the low-tide period, the fish should be hungrier and thus stimulated to feed (Burrows *et al.,* 1999). A study conducted on intertidal fishes on the west coast of France showed that *L. pholis* could move down shore into deeper waters during the winter months (Gibson 1967b). Furthermore, the refered study was conducted both in intertidal and subtidal areas, and the decrease in the numbers of fish during the winter months was observed in all habitats. Thus, it seems likely that this decrease could be related to a general reduction in feeding condition of these fish at low temperatures with fish being more restricted to shelters like pools or crevices in the rock platform, and hence less easy to detect by observers (Faria & Almada, 2006). This interpretation is also supported by an observed a decrease in the feeding frequency of these fish at low temperatures (Qasim 1957). According to these, during the winter, there is a significant decrease of abundance of *L. pholis* in northwest Europe (e.g. Gibson 1967b, Milton 1983; Dunne 1977), including in Portugal (Faria & Almada, 2006).

L. pholis can live in a variety of substratum types during its life cycle. Juveniles occur in shallow pools of simpler topography (Fig. 2[A]), but when they grow they tend to leave the pools and begin to seek larger pools (Fig.2 [B]), crevices (Fig. 2[C]), spaces under stones and other protected microhabitats (Qasim, 1957; Faria & Almada, 2001b). Both juveniles and adults clearly prefer rocky to sandy substrata. It is likely that this preference could represent a better protection from both predators and waves action (Almada & Faria, 2000).

In Portugal, a study of microhabitat segregation (i.e. removal experiments) in *L. pholis* (Faria & Almada, 2001a) suggested that in every year, each pool is occupied by the number of fish of each size class that it can shelter. Therefore, migration may just be inevitable, with 70-80 mm being the limiting size for the adequate usage of 'higher' or very shallow tide pools. These rock pools, presenting a complex substratum, with numerous small holes and crevices, and generally low water volumes, could provide higher protection from predators during the low tide, but are only suitable for small fishes. Probably due to specific mechanisms of rock erosion on the intertidal platform, the number of hiding places suitable for larger fishes is scarce in these rock pools (Monteiro *et al.,* 2005) forcing individuals above a certain threshold to migrate downwards to larger pools, crevices and channels as observed not only for *L. pholis*, but also for some gobiid species (Faria & Almada, 2001a)

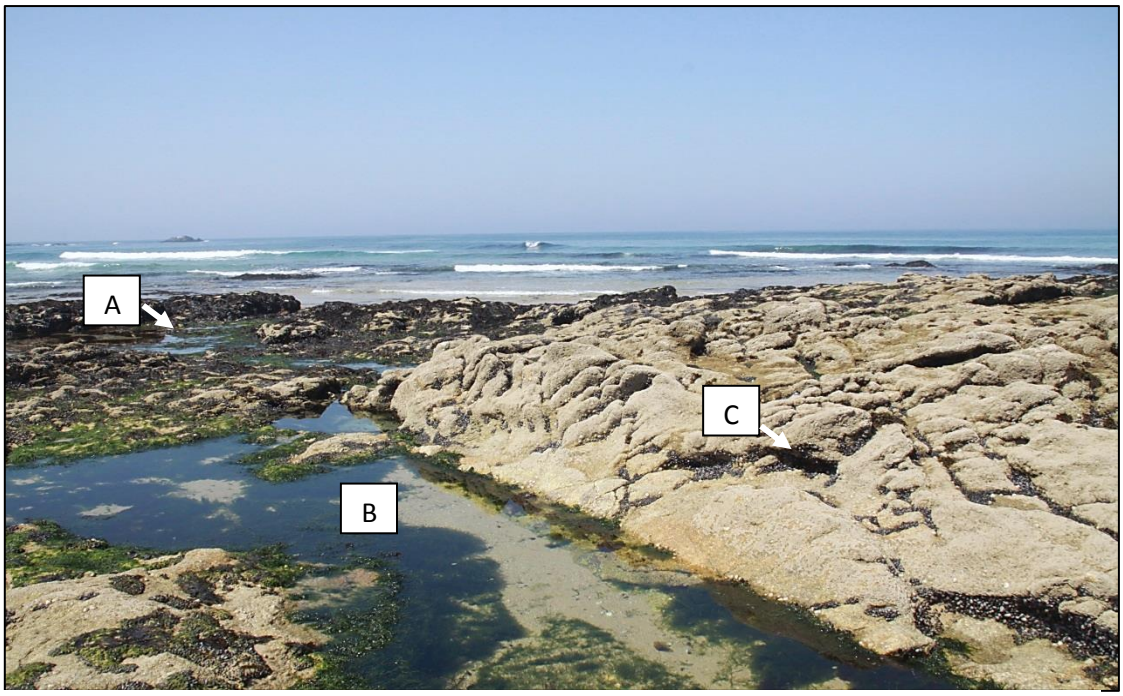

Fig. 2- Rocky beach representing the [A]) shallow pools of simple topography where juveniles occur; [B]) larger pools and [C]) crevices, were fish > 7 cm occur. Personal capture.

The intertidal fishes show patterns of courtship and agonistic behavior that minimize the loss of contact with the substrate, a feature that is probably adaptive in conditions of marked turbulence (Almada & Santos, 1995). Juveniles of *L. pholis* could defend the access to shelter holes existent within their home range habitat, but without exclusive use (Gibson, 1968). Usually *L. pholis* use a network of familiar pathways that included more than one shelter (Almada *et al.,* 1983). The individuals would compete not for a permanent presence in a shelter, but for undisputed priority of access to the shelters present in the neighboring areas, to minimize the time during which the fish is exposed to potential predators. In the case of the breeding males, the "diffuse territoriality" would change to a more traditional form of territorial defense, because they tend to concentrate their visits on a single hole and restrict their movements to the surroundings areas. The findings that fish removed from pools and released several meters away were found in their original pools support the hypothesis that when in pools, fish acquire information on the exact locations of shelters (Qasim, 1956a; Almada *et al.,* 1983, 1990b; Almada & Santos, 1995).

Some rocky intertidal fish have been shown to have good homing abilities (Gibson, 1967; Santos *et al.*, 1989; Mitamura *et al.,* 2005) and believed to have mapping orientation abilities (Arondson, 1951; Gibson, 1968; White & Brown, 2014). Homing behavior has been generally defined as the ability of an animal to return to a spatially restricted location that it previously occupied following displacement to an unfamiliar site (Williams, 1957; Gerking, 1959; Papi, 1992). One of the key benefits of homing is that it ensures an animal returns to a familiar location (White & Brown, 2013). Homing is likely to be particularly important in extreme environments, where conditions fluctuate greatly over space and time. In intertidal fish, for example, survival may be influenced by an individual's ability to return to its home rock pool after feeding excursions took place during the high tide periods and thereby avoid being stranded in unsuitable areas at low tides (Williams, 1957). Adults of *L. pholis* were able to orient themselves toward their home areas even if they were placed in an unfamiliar distant area by relying on cues available in the unfamiliar area (Jorge *et al.,* 2012) They can easily find a refuge, in a novel habitat by quickly swimming toward the nearest dark area (Dodd *et al.,* 2000) and are able to memorize it using spatial maps of local landmarks (Burt de Perera & Guilford, 2008).

In a recently published study, two hundred and eleven *L. pholis* fish were tracked over a three-year period using tiny electronic tags (Jorge *et al.,* 2016). Results showed that *L. pholis* males found its way back to the same nest to tend to its eggs year after year which is surprising to see in a non-migratory species standard behaviors of a migratory one. Once as juveniles and adults *L. pholis* remain resident in isolated rocky intertidal areas, the connectivity

among populations appears to depend entirely on the oceanic dispersal during the planktonic larval stage (Francisco *et al.,* 2006).

Morphology, Systematic relationships and Phylogeny

Several authors have described the developmental life stages of *L. pholis,* including brief descriptions of the eggs and larvae. Hefford (1910) presented a brief description of the pigmentation of a larva 4.4 mm Total length (TL), which is probably a developing embryo that hatched precociously. Lebour (1927) provided a detailed description of the pigmentation of the newly hatched larva (5.4 mm TL). Ford (1922) presented a brief description of the pigmentation of larvae 5.0 mm, 5.5 mm, 9.0 mm and 17.5 mm TL. McIntosh (1905) described the pigmentation and morphology of post-settlement individuals (TL > 19 mm). Faria *et al*, (2002) described through captive experiments the full developmental sequence of *L. pholis* from the eggs to juvenile stage. *L. pholis* eggs are golden-brown, transparent with a spherical shape (Fig. 3) except at the attachment disk.

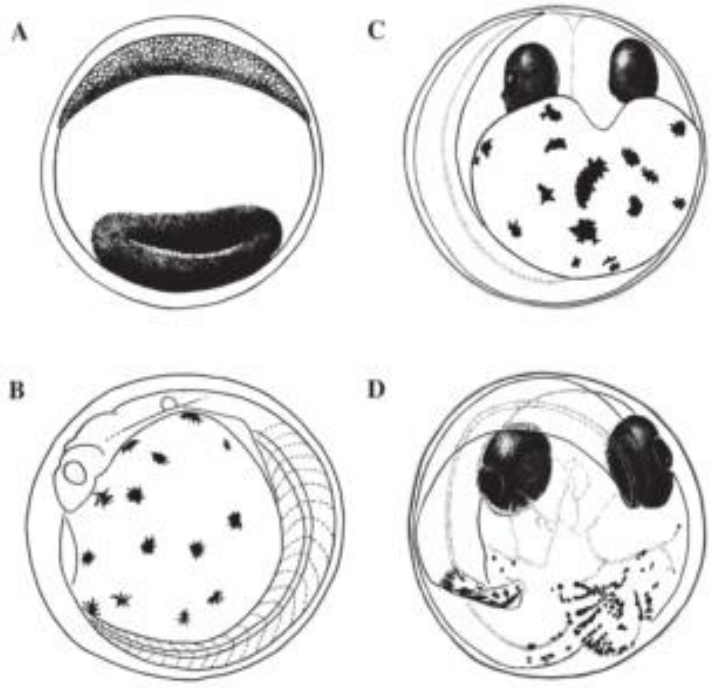

Fig. 3 - Eggs in different developmental stages: (A) Day 1; (B) Day 5: Embryo almost reaching the margin of the yolk; (C) Day 8: embryo longer than egg major axis: (D) Day 15: embryo prior to hatching (dorsal view) (From Faria *et al*., 2002)

The newly hatched larvae present fully pigmented eyes (Fig. 4), with the anus and mouth already open. At hatching larvae present peritoneal pigmentation and twelve rows of melanophores on the pectoral fins. Ventrally there were 2-4 melanophores on the throat and 7-9 on the last myomeres. Dorsally, there were some sparse melanophores over the brain and upper lip and there was one melanophore between the inner ear vesicles

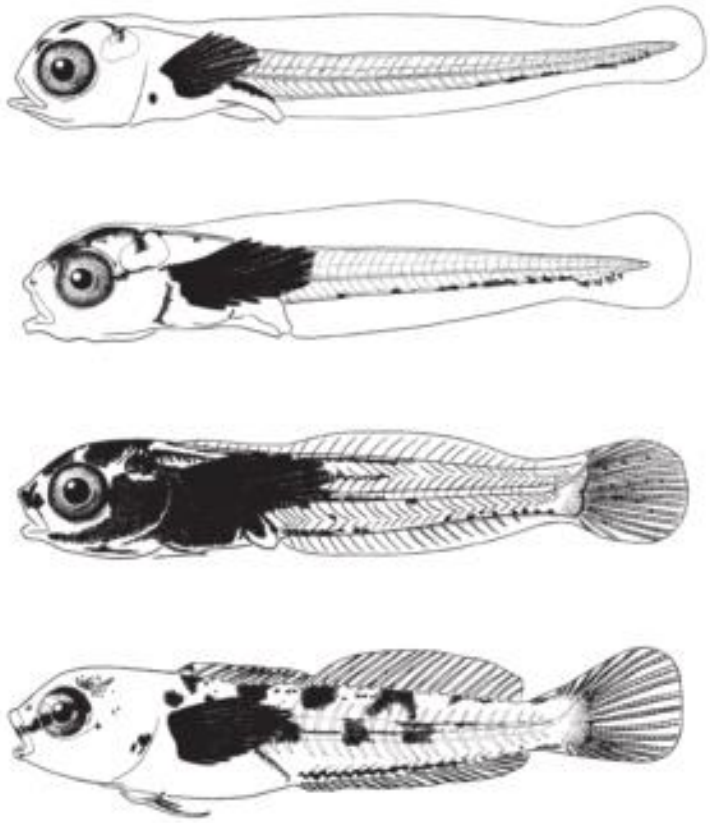

Fig. 4- Larvae in different developmental stages: (A) Day 1: newly hatched larva (5.5 mm TL); (B) Day 5:6.3 mm TL; (C) Day 25:13.0 mm TL; (D) Day 41: juvenile (17.0 mm). (From Faria *et al*., 2002)

After metamorphosis the juvenile fish developed the final pigmentation. A ventral row of melanophores at the base of the anal fin was present and the other fins were also pigmented. The head was extremely pigmented and there was some pigmentation at the throat. Dorsally there were dark bands that extended through the midline, and alternated with three other blotches situated laterally (on each side of the body). The pigmentation pattern is maintained during development with an increase in the number and intensity of melanophores at the ventral row, and at the cephalic region, with melanophores extending from between the eyes to the dorsal region (Fig. 4).

The embryonic developmental sequence described for Faria *et al,* (2002) generally agrees with Qasim (1956), except the timing of the life history events that occurred earlier. While Qasim (1956) recognized the differentiation of the embryo at day 8 after hatching, the presence of eye rudiments at day 14, and the formation of myomeres and heart beatings at day 24, Faria *et al,* (2002) observed these events at day 2, day 4, and day 5 respectively.

In Faria *et al,* (2002) study, embryonic development lasted 16 days at 17ºC, while Qasim reported an embryonic developmental time of 43 days at 11.5-15.0ºC, and 61 days at 9.5-14ºC. These differences are probably due to the incubation temperature since the decrease of the developmental time with higher temperatures is known for many fish species (Blaxter, 1969). Nevertheless, the difference of almost 50% in the timing of developmental events is noteworthy (Faria *et al.*, 2002).

L. pholis adults has morphology characteristics typical of other blennies (Fig. 5), such as a smooth and elongated body, with a large and blunt head, fairly large eyes and small tentacles bellow the eyes, but without head tentacles and its eyes are set high up on its head (Zander, 1986; Arruda, 1979).

Fig. 5- Photography of an adult *L. pholis (personal capture).*

McIntosh (1905) described ways of distinguishing the two sexes using the body pigmentation and the shape and the position of genital papilla and aperture. The best distinguish character, however, is the shape of the head in the adult's individuals. Males have a prominent bulging forehead, and the females the head is much flatter. Ford (1935) remarks on the fleshy head which curves sharply towards the mouth, but this character is fully developed only in the male. The bulging is more pronounced in those males which are

approaching maturity. Dissections of *L. pholis* male revealed a mass fat under the skin of the bulge, possibly this act as a reserve of food during the period of incubation by the males, when little feeding occurs (Qasim 1956).

Ferreira *et al.,* (2010) described the genital morphology of papilla and ano-genital distance for adult's specimens. The male's genital papilla was located about midway from the anus to the first soft anal ray and was characterized by a globular protuberance, swollen during the breeding season, with the genital pore located near the tip. The female's genital papilla, however, was located closer to the first soft ray, which was largely embedded in dermal tissue, with only the tip of the ray remaining visible. In females, the broad genital opening was located on the external surface of the ray, delimited by a skin fold and should not be confused with a cavity that is located between the two soft anal fin rays. The ano-genital distance is highest in females and distances tend to diminish during the breeding season. During spawning, the males present a general black coloration with white lips and presence of dorsal club glands on the tips of dorsal fin rays (Ferreira *et al.,* 2010), while the females show a light coloration, with fins almost transparent (Qasim, 1956; Faria *et al.*, 2002).

L. pholis is phylogenetically closer to *L.* (= *Paralipophrys*) *trigloides* and to *Coryphoblennius galerita* than to the other small *Lipophrys* species, which form their own independent monophyletic group (Bock & Zander, 1986; Almada *et al.,* 2005). A close relationship between *L. pholis*, *L. trigloides* and *C. galerita* has already been proposed by other authors based on osteological and karyological data (Papaconstantinou, 1977; Bock & Zander, 1986; Garcia *et al.,* 1987). Data from molecular studies found that *L. trigloides* and *L. pholis* are sister species, while other small *Lipophrys* species formed a very distinct clade, questioning the monophyly of the genus *Lipophrys* which, according to the authors, must include only *L. pholis* and *L. trigloides* (Almada *et al.*, 2005). A mitochondrial DNA study of *L. pholis* found no significant population genetic structure along the Portuguese coast, attributing this genetic homogeneity of the shanny to efficient gene flow (Francisco *et al.*, 2006). However, Stefanni *et al.,* (2006) verified the presence of two groupings of shanny in NE Atlantic, one for the Azores and one for the mainland Europe which includes the island of Madeira.

Feeding ecology

Several quantitative and qualitative studies about the dietary habits along its geographical distribution exist during the ontogenetic development of *L. pholis* (Qasim, 1957; Dunne, 1977; Carvalho, 1982; Milton, 1983; Wyttenbach & Senn, 1993; Mazé *et al.,* 1999). Apart from particular differences, all findings converge on the generalist nature of this species that

forages on heterogeneous diet. All studies agree on the importance of mussels, barnacles and limpets as major components of the diet of this species.

A study in a rocky beach located in the north of Portugal showed that some prey items, important for the smallest size classes, tended to disappear from the diets of larger individuals, being progressively replaced by different prey items (Monteiro *et al.*, 2005). In the diet of the smallest *L. pholis* (classes 0-40 mm and 40-50 mm), a contribution of small prey was visible, such as *Skeneopsis planorbis*, Neogastropoda, Isopoda, Amphipoda, Copepoda and other Mesogastropoda. As *L. pholis* grows, there was an increase in the relative importance of sessile preys. Adult individuals (>80 mm) tend to eat large quantities of *Mytilus galloprovincialis*, *Patella* spp., *Gibbula* spp., and other Archaeogastropoda and Polyplacophora. Older individuals also tend to eat large quantities of red and green algae (*Osmundea pinnatifida* and *Ulva sp*., respectively). In the stomach of large *L. pholis* a considerable quantity of *Chthamalus spp*. was sometimes observed, still interconnected, suggesting that these fish scraped the rock, ingesting large clutches of barnacles (in some cases, several *Melarhaphe neritoides* were still visible inside empty barnacle plates) (Monteiro *et al.*, 2005).

The change in the feeding regime may be related to when juveniles of *L. pholis* reach a total length of 70 mm and initiate a radical shift in their pattern of microhabitat occupation, to migrate downwards to larger pools, crevices and channels, living the tide pools (Faria & Almada, 2001a). Thus, reproduction, diet change and microhabitat shift of *L. pholis* appear to be intimately related to explain their distribution in a rocky beach. A more energetically profitable diet, preyed upon with considerably lower effort (mainly abundant sessile organisms), might be essential to compensate and maximize the reproductive effort.

In Spanish Cantabrian coastal waters, the *L. pholis* diet revealed that during Winter the diet richness is poor, with only 24 prey taxa recorded. Diet species richness showed a considerably increase in spring and also during the Summer and Autumn months (37 taxa). This period match with the settlement of many species of invertebrates which, moreover, have the appropriate size to be ingested by the blenniids (Mazé *et al.*, 1999). However, some variations in feeding time and habits occur during the breeding season in parental males. *In situ* observations in a Portuguese rocky beach showed that the daily average feeding time of *L. pholis* male parents is relatively short (5 h and 34 min) (Almada *et al.,* 1992) which corresponds to the daylight time period when territories are underwater. Since blenniids tend to be diurnal fishes this is probably the maximum amount of time in which feeding and all other activities performed outside the nest are possible. Since 92% of this time is spending inside the nest, feeding is restricted to about 27 minutes per day (Almada *et al.*, 1992). Such a

feeding restriction associated with parental care has been documented for the males of some gobiids probably due to reduced feeding and/or high energetic demands (Miller, 1984; Magnhagen, 1986, Santos & Almada, 1988). Furthermore, the defense of food resources may be essential for the survival and breeding success of the parental males of *L. pholis* in a period when parental care restricts their movements to a very small area (Almada *et al.,* 1992).

In British islands, Qasim (1957) described that food of male parent fish exhibit some interest peculiarities. Examination of males collected in the earlier months of the breading season, showed their guts empty or with very little food while those collected in the later part of the breeding season showed normal guts with high food concentrations inside (ex: algae, hydrozoa and mollusks). However, those guts contained mainly shanny eggs, presumably taken from egg masses they were guarding. This habit of eating eggs and larvae of their own was already described often been in aquaria observations (Qasim 1956). The composition of the parent's food therefore suggests that availability of organisms around a guarded area is more important than unrestricted choice of the fish. Whenever the demand for food seems to be great, they begin to eat their own eggs (Qasim 1957). In Portugal the habit behavior of *L. pholis* of eating own larvae or eggs were not recorded. Almada *et al.,* (1999b) through field observations described, however, that parental males have a particular and interesting cleaning behavior like removal of air from the nest, debris and small objects (including dead eggs) with the mouth.

Age and Growth

In Portugal, field observations show that six months after the strongest recruitment of *L. pholis* (april to june), the modal class was 50-60 mm long for fishes with 0 years old (Faria *et al.,* 1996). Nonetheless, Qasim, (1957) reports a mean size of 45.5 mm for 0 years fishes 6 months after recruitment, which suggests that growth of 0 years fishes is faster in Portugal than in Britain as well as for older years classes (Faria *et al.,* 1996).

Dune, (1977) study *Lipophrys pholis* in Irish waters and concluded that both males and females appear to grow at the same rate. The oldest specimen collected was a female entering fourteenth spring of life with a total length of 182 mm The oldest male was eleven years of age and measured 170 mm however a survival curve were constructed from the relative frequency of age groups and *L. pholis* has a potential lifespan of thirteen or fourteen years, but only a small fraction of the population lives more than four years. A similar mortality rate was recorded in Menai Straits (Qasim, 1957). The reason for a high mortality rate during the early years of life is not clear. The number of suitable habitats probably decreases as

specimens grow larger, consequently there is probably competition for space among larger specimens. The mean total length of each group demonstrated that growth is rapid in small immature specimens but slows down after a few years when sexual maturity is reached (Fig. 6) (Dunne, 1977)

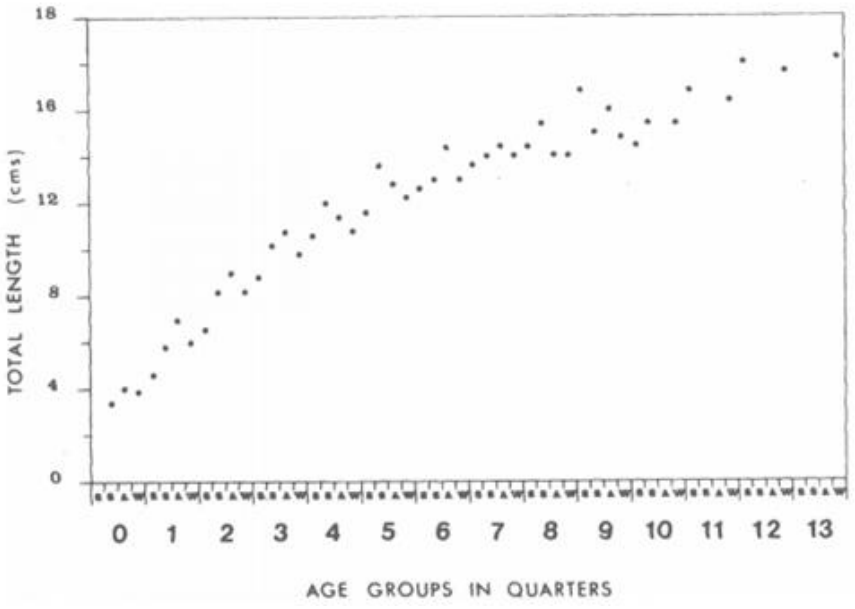

Fig. 6- Mean total length in each quarter. (From Dunne, 1977)

Growth follows a similar pattern at Menai Straits (Qasim, 1957), and in *Gocius paganellus* (Miller, 1961), *Gobius cobitis* (Gibson, 1970) and *Throgobius ephippiatus* (Dunne, 1976). The growth rate at Carna is slower than at Menait Strait or the Isle of Man (Fig.7) and the oldest specimens are also found at Carna. Qasim (1957) did not record specimen older than 6^+ at Menai Strais, Bowers *et al.,* (1960) recorded a specimen of age 10^+ at the Isle of Man, at Carna (Dunne, 1977) the oldest specimen was 13^+ and a few specimens were more than 10^+. In Portugal the oldest specimens recorded was 3^+ of age at Cabo Raso and Arrábida with a mean size of 125.3 mm (Faria *et al.*, 1996).

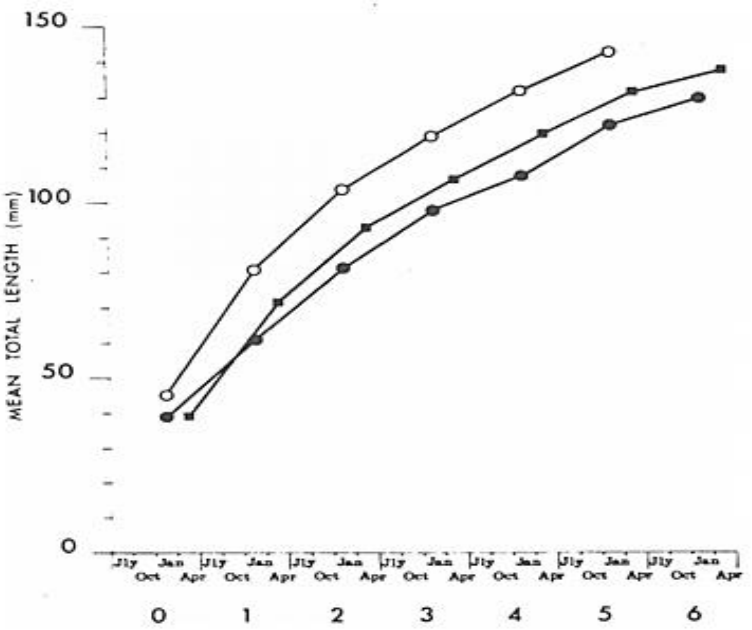

Fig. 7- Comparisons of growth rate of *L. pholis* from the Menait Straits (open circle); Isle of Man (square) and Carna (closed circle). Data from the Menai Straits after Qasim (1957); data from the Isle of Man after Bowers *et al*., 1960. (From Dunne, 1977)

In the Atlantics Islands, there is a well-known phenomenon of gigantism where the individuals may reach more than 300 mm TL in Azores. Large sized individuals are also found at Madeira, when Fowler (1936) recorded an individual at 305 mm TL. The individual sizes found at Madeira and the Azores are almost the double of the maximum individual sizes found in European populations of the species (Santos *et al.,* 1988).

Reproductive Biology

In Great Britain *L. pholis* spawning takes place during Spring and early Summer (March/April to August) (Qasim, 1957). In the Portuguese coastal waters, *L. pholis* breeding season occurs earlier from early Autumn to middle Spring (October/November to May) (Faria *et al.,* 1996), which supports the findings that southern fishes will spawn in the cooler months of the year at the southern limit of their range (Qasim, 1956). Furthermore, the breeding season of *L. pholis* appear to be longer with the decreasing in latitude, and nests containing eggs can be easily observed from early October to late May (Faria *et al.*, 1996).

Intertidal fish species, such as blenniids, usually use cavities as nests were spawning takes place and male guard the demersal eggs (Qasim 1957, Almada *et al.,* 1992) (Fig. 8)

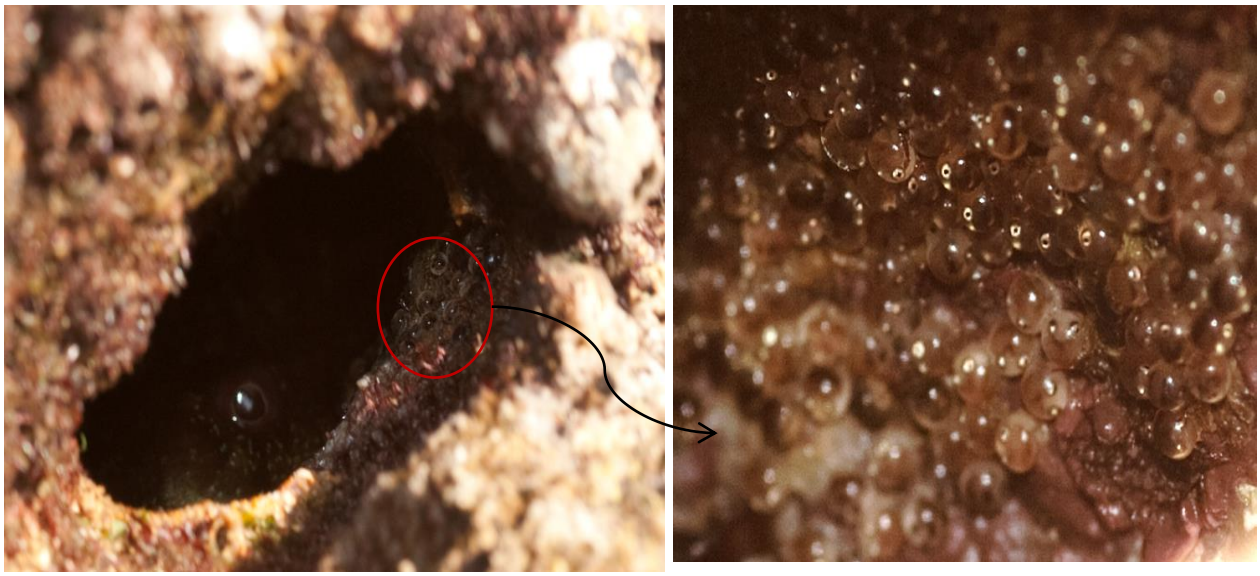

Fig. 8 – Male *L. pholis* in a crevice guarding the eggs. *Henrique Queiroga capture*.

Captive experiments (Faria *et al.,* 2002) showed that spawning lasted more than 9 h. The female was over the nest wall and the male approached, touched the female with the snout and rotated until the genital papilla touched the female's back. After touching the female, the male performed pectoral fin beatings and high amplitude movements of the tail and posterior part of the body, rubbing the nest wall with the genital papilla. This movement ended with a brief body shaking. Following the male's path, the female applied the belly to the nest wall and skimmed over the nest surface with slowly pectoral fin movements while quivering the tail. The genital papilla touched the nest wall several times with the eggs being laid one at a time in a single layer. This sequence was repeated several times, alternating with resting periods. In general, both fishes alternated their movements over the stone (Faria *et al.*, 2002). Those observations from Faria *et al.,* (2002) of spawning behavior contrast with that provided by Qasim (1956) in an important detail. While Qasim's description implies that the male fertilizes the eggs after attachment, Faria *et al.,* (2002) observations based on videotape recordings point to the contrary. The male first rubs the substratum with the genital papilla and the female follows the male's path while laying eggs, suggesting that the female spawns over a surface that is likely to already contain sperm. Patzner (1984) showed that the micropyle of the eggs of blenniids is in the middle of the adhesion disc and thus faces the substratum when the eggs are attached. Qasim (1956) reported that in the ovary, the position of the eggs is such that they must be extruded with the adhesion disc facing the substratum. This means that it is very likely that contact with sperm must precede attachment, either through the presence of sperm in the water column or by a sperm layer previously attached to the rocks by the male, as

decribed for some gobiids (Marconato *et al.*, 1996; Ota *et al.*, 1996; Faria *et al.,* 1998). Faria *et al.,* (2002) observations suggest that the male probably applies sperm to the rock surface before egg attachment.

Field observations, show that after spawning occurs the male defend and ventilate the developing eggs until hatching and guard multiple clutches, from single or different females (Almada *et al.,* 1990b). *L. pholis* males revealed to have agonistic behavior during breeding season. The active defense of the area around the nest probably serves, at least, two functions - keeps potential egg predators and other intruders away from the nest; and minimizes feeding activities of conspecifics in the area surrounding it (Almada *et al.,* 1992).

The pelagic larval development lasts 29 days at a temperature of 15.5 - 17.5°C (Faria *et al.,* 2002). During spring, when they reach a total length of 13-20 mm (Faria *et al.,* 1996, 2002), the recruits *L. pholis* start to settle to intertidal pools. Faria et al., (1996) found a longer period of recruitment for *L. pholis* in Portugal compared to higher latitudes, with the first juveniles being present in the intertidal pools as early as January. By September – October, with a size of approx. 60-70 mm, the juveniles begin to colonize adult habitat (Monteiro *et al*., 2005). Recruits were considered to be all fish ≤ 30 mm TL (Faria & Almada, 1999), from 30 - 60 mm TL were considered as juveniles and fish > 60 mm TL as adults (Faria & Almada, 2001a). According to Faria *et al*., (1996), fishes with 1 year proved to be mature upon gonadal macro inspection. Three males and six females were collected, during the spawning season, in a rocky beach in west coast of Portugal higher latitudes, previous studies report that both sexes mature when they are 2–3 years old (Qasim, 1957; Dunne, 1977; Milton, 1983).

The basic patterns of oogenesis and spermatogenesis in *L. pholis* are all similar for that described for other blenniids (Qasim, 1957; Dunne, 1977;, Shackley & King, 1977; Fives, 1980; Patzner, 1983; Santos, 1995; Carrassón & Bau, 2003) or even to other teleosts (Tayler & Sumpton, 1996; Lubzens *et al.,* 2010). Qasim (1957) described five maturation stages based on color, shape, size and weight of the gonads (not based on histological procedures): immature virgins; maturing virgins or recovered spents; ripening; ripe and spent.

Ferreira *et al.*, (2012) studied the oogenesis of *L. pholis* (only in mature individuals, larger than 8 cm) in Portuguese rocky beaches (Fig. 9). Macroscopically, the ovaries of *L. pholis* are paired, bilobate organs located in the celomic cavity. During maturation, ovaries increase in mass and broadness and colour becomes more vivid. Covered by a thin, highly vascularized membrane. Through histological observations, seven types of ovarian germ cells were identified during oogenesis: oogonia, early perinuclear oocytes; late perinuclear oocytes; cortical-alveolar oocytes; early vitellogenic oocytes; vitellogenic oocytes; and spawning

oocytes. Thus, according to both the macroscopic characteristics and the frequency of ovarian components, 3 stages of maturation were defined: early oogenesis, mainly observed in May; mid-oogenesis, mainly observed in September and spawning occurring between November and January. The highest gonadossomatic indices (GSI) values were observed in January (GSI=4.24) while the lowest was observed in May (GSI=1.19). A positive correlation occurred between female size, female weight, spawning oocyte diameter and gonad weight, which suggest that larger female have the potential to produce larger broods (Ferreira *et al.,* 2012).

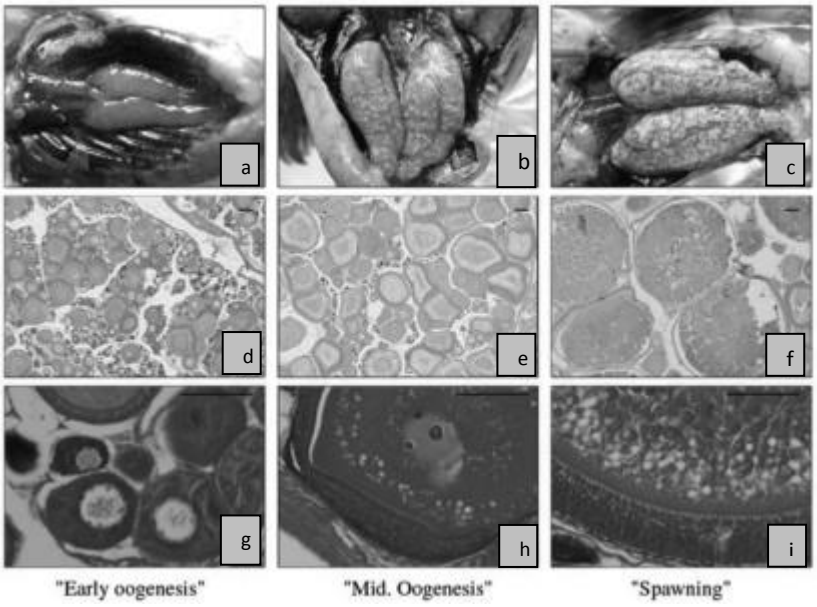

Fig. 9 – Macroscopic photos of ovarian gonadal development [(a) earl oogenesis; (b) mid-oogenesis; (c) spawning]. Histological architecture and photomicrographs of transverse sections of *L. pholis* ovaries within the three considered maturation stages also depicted [d, g, early oogenesis; e, h, mid oogenesis; f, i, spawning]. d, e, f scale = 500 mm; g, h, i scale = 50 mm. (From Ferreira *et al*., 2012).

Similar trends were observed in the British Isles (Qasim, 1957; Shackley & King, 1977). Nevertheless, in these more northern populations, although fish size [Qasim, (1956) reports a maximum female size of 157 mm] and gonad weight [Shackley & King, (1977) report a weight of 1.8 g] can be considered similar to those observed in Ferreira *et al.*, (2012), the diameter of spawning oocytes seems to differ. While along the Portuguese coast the maximum spawning oocyte recorded was only 0.92 mm (average of 0.81 mm: Ferreira *et al.*, 2012), Shackley & King, (1977) report a size of 1.35 mm for the British Isles.

According to the results of Ferreira *et al.,* (2012) considering fish size and spawning oocyte diameter, an oocyte this big would have been laid by an equally large female (260 mm). Alternatively, it seems more parsimonious to infer that more northern populations may have a distinct investment pattern in reproduction in comparison with those that live near the species

southern limit of distribution. Given that gonad weight is similar but spawning oocytes are smaller, it also seems reasonable to infer that females of southern populations, through a broader breeding season (Faria *et al.,* 2002), disperse their total reproductive investment over a longer time period (Ferreira *et al.*, 2012).

All stages of development were represented in the ovaries during all seasons suggesting that *L. pholis* is an asynchronous spawner, with eggs being recruited in several batches during the breeding season. It is possible that the asynchronous production of multiple batches functions as a bet hedging strategy, allowing the eggs to be distributed among several males, thus reducing the risks of complete loss of progeny because of inadequate mate choice, environmental constraints and failure in larval recruitment, among other equally valid causes (Qasim, 1957; Ferreira *et al.,* 2012).

Regarding to male gonadal maturation, Qasim, (1957) described five maturation stages based on color, shape, size and weight of the gonads: immature virgins; maturing virgins or recovered spents; ripening; ripe and spent. Testes are similar for that described for other blenniids (Santos *et al*., 1995) through visual identification, male gonads testes are paired elongated bodies and consisting of tubules of the unrestricted spermatogonial type. When immature their color is transparent, but when mature their color is milky white. After spawning the gonads become much shrunken and greyish. Male gonads are composed of two main components: the testis and the testicular gland. It has connection to the tubules and the vas deferens (Santos *et al.*, 1995; Qasim, 1957).

Ferreira *et al.*, (2012) studied the spermatogenesis of *L. pholis* (only in mature individuals, larger than 80 mm) in Portuguese rocky beaches (Fig. 10) and through histological observation characterized the different testes cell types: Spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids were usually located at the periphery of the lobules, while spermatozoa appeared, as free elements, in the center of the lobule. The seasonal changes in the testes were defined based on the histological characteristics and on the relative abundance of spermatogenic cell types. According to the frequency of testicular components, three stages of gonadal maturation were defined. Early spermatogenesis, mainly observed in May, mid-spermatogenesis, mainly observed in September and spawning occurring between November and January (Ferreira *et al*, 2011). The highest GSI values were recorded in January (GSI=1.13) while lowest values were observed in May (GSI=0.33).

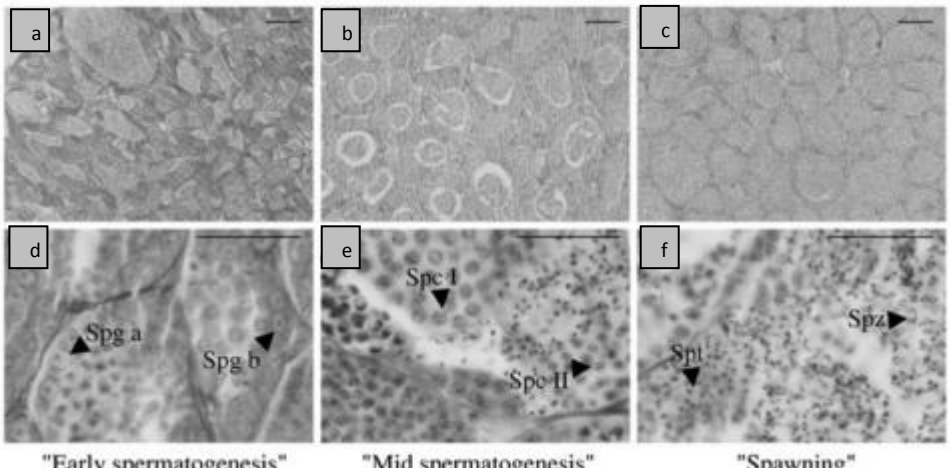

Fig. 10- photomicrographs of the histological architecture of L*ipophrys pholis* testis: (a early spermatogenesis, b mid spermatogenesis, and c spawning). Additionally, the microscopic morphology of the spermatogenic cells are also depicted: d type A spermatogonia (Spga) and type B spermatogonia (Spgb), e, primary spermatocytes (SpcI) and secondary spermatocytes (SpcII), and f, spermatids (Spt) and spermatozoa (Spz). The scale for a, b and c represents 200 μm while for d, e, and f, it represents 20 μm. (From Ferreira *et al*., 2011)

Given the fact that spermatogonia were always observed throughout the year, namely during the breeding season, it seems that *L. pholis* males are capable of multiple spawning episodes, as already observed for other Blenniidae (Qasim, 1956; Shackley & King 1977; Carrasson & Bau, 2003). Interestingly, Shackley & King, (1977) observed that *L. pholis* females are also capable of spawning several times during the breeding season.

Although the number and characterization of *L. pholis* maturation stages varies in the available literature (Qasim 1957; Shackley & King 1977), it should be noted that the previous studies relied on methodologies based on macroscopic observations. Qasim, (1957), as well as Shackley & King, (1977), described an additional ‘‘spent’’ as well as a ‘‘resting’’ period. Since, for the same maturation period, the maximum weight of *L. pholis* gonads was considerably different in Portugal (0.23 g; Ferreira *et al.,* 2011) when compared with individuals from higher latitudes (0.67 g; Qasim, 1957; Shackley & King 1977). It is possible that this species presents reproductive investment strategies that vary according to latitude (Conover, 1992): a higher investment in higher latitudes, where the breeding season is shorter, thus explaining the observed ‘‘spent’’ and ‘‘resting’’ period, and a less pronounced, but more expanded investment in the south. Alternatively, the difference in gonad weight might result from different levels of sperm competition that might arise from the extent of the breeding season, which largely differs at contrasting latitudes (Ferreira *et al.,* 2011)

Qasim, (1957) reported for this species, the condition factor (K) reach peaks in June which corresponds to the period of gonad ripeness. Minimum values of K in both sexes were obtained between July and September, which coincide with the end of spawning.

1.3 Otolith microstructure and microchemistry as an useful ichthyological tool

Otoliths (earstones) are paired calcified structures used for balance and/or hearing in all teleost fishes that play an important role in balance and auditory reception of teleost fish (Popper & Platt, 1993). The calcium carbonate ($CaCO_{3)}$ is crystallized mainly in the mineral form of aragonite (Carlström, 1963) due to the action of an organic matrix (otolin) in which acidic amino acids predominate (Degens *et al*., 1969). The inner ear in fishes is typical of that of other vertebrates, having three semicircular canals and three otolithic organs.The semicircular canals and associated ampullae detect angular accelerations, whereas the otolithic organs appear to have a dual function, vestibular and auditory (Popper & Lu, 2000). In the labyrinth of teleosts there are generally three different otoliths (sagitta, lapillus and asteriscus), each having an irregular unsymmetrical shape which is characteristic of each species. The sagittae and lapilli generally form earlier in development than the asterisci, which in some species do not form until after hatching (Green *et al.*, 2009). They are located in the otolithic organs, respectively in the sacculus, utriculus and lagena (Carlstrom, 1963). From the three pairs of otoliths, the sagittae are usually the largest and most common otoliths used for microstructure studies (Campana & Neilson, 1985). Based on the ability of the otoliths to record the life history of the fish, they are extensively used in several types of study of fish biology and fisheries science, namely for ageing purposes (Secor *et al*. 1995a). Over 1 million fish were likely estimated through otoliths by fisheries scientists around the world (Campana & Thorrold, 2001).

Age determination of fishes based on periodic growth increments in otoliths has become a routine tool in fisheries science over the last century. Reibisch, (1899) was the first to observe and use the translucent and opaque rings to determine the annual age of fish.

Opaque zones are extensive areas of rapid growth, corresponding to the large deposition of aragonite during the summer warm months and, translucent zones, are narrow areas of winter slow growth, corresponding to the lower deposition of aragonite during the cold months (Hunt, 1980). Since Reibisch's observations of the annular ring formation otolith (*annuli*) in *Pleuronectes platessa,* there has been a growing interest in the use of the otolith as an

indicator of annual age. Annual ageing is often used in support of harvest calculations and populations studies, and can be based on any bony structure in the fish, although scales and otoliths are the structures most frequently used (Campana & Thorrold, 2001). In contrast, daily ageing based on the otolith microstructure tends to be targeted more at recruitment questions and studies of larvae and young fish (Campana & Neilson, 1985). Although the use of otolith age fish is one of the most common and spreading uses of these crystalline stones, today the applications of the otoliths go far beyond the simplest classical age calculations (Campana *et al*., 2000).

Daily growth increments in calcified structures are restricted to species in which the depositional environment of the structure can be controlled by the organism without subsequent resorption (Campana and Thorrold 2001), and includes bivalve shells (Richardson 1988), squid statoliths (Jackson 1990) and fish otoliths (Campana and Neilson 1985). Daily growth increments in otoliths of teleost fish are now known to be a widespread phenomenon, present in taxa in both freshwater and marine habitats, and in species distributed from the polar regions to the tropics (Campana and Neilson 1985). Individual otoliths can be analysed to provide a daily record of age and growth rate with high accuracy and precision (Campana 1984).

The otolith daily growth increments result from an endogenous circadian rhythm of increment formation, entrained by photoperiod (Campana and Neilson 1985), but susceptible to modification by other cyclic environmental variables, like the temperature and feeding regime (Campana and Neilson 1982; Neilson and Geen 1982; Alhossaini and Pitcher 1988). Otoliths grow by successive deposition of increments, the so-called primary or micro increments, which constitute bi-partite structures each composed of one L-zone and one D-zone (Mugiya et al. 1981). The L-zone is a band rich in calcium carbonate crystals, translucent to light (LM) and appearing raised in scanning electron microscopy (SEM), with an increment width varying between 0.4 and 10 µm. The D-zone is a band rich in organic material, opaque to LM and appearing as a groove in SEM, with an increment width smaller than 1 µm (Panfili *et al*., 2002). The width of a primary increment (also named micro-increment) usually ranges between 1 and 12 µm (Pannella, 1974). Micro-incremental patterns in the otoliths vary from sub-daily to daily, lunar and seasonal scales (Campana & Neilson, 1985). Rhythmical patterns in the deposition of increments in the otoliths of fish are the basis of age estimation and depends of an endogenous rhythm externally calibrate by the photoperiod (Morales-Nin, 2000). The influence of lunar rhythms in the recruitment of coral reef species has been demonstrated since it alters the otolith microstructural pattern (Sponaugle & Pinkard, 2004). Environmental conditions affect the otolith growth rate (increment width) but increment

periodicity may be disrupted in extreme cases of physiological stress (Morales-Nin 2000). In fact, some works showed that the daily rhythm of the increment formation continued in fish held under constant light (Campana 1984), darkness (Radtke & Dean, 1982) or in absence of cyclical variations in other major environmental factors (Wright *et al*. 1992), although the effects of environmental conditions on increment formation vary among species (Jones, 1986). On the other hand, once deposited, the calcium carbonate of the otolith is reabsorbed only in extreme stress (Mugiya & Uchimura 1989). Food intake and food deprivation also have an influence on microstructure, width and periodicity of increments in otoliths (McCormick & Molony, 1992; Molony, 1996; Massou *et al*., 2002). Stress-induced marks indicate the cessation of otolith growth, which is rare phenomenon, and appear under transmitted light microscopy as opaque, regular, thin marks (Pannella, 1980).

The rate of formation of otolith growth increments theoretically permits age determination with high precision, often to the daily level (Campana and Neilson 1985). Although the apparent rate of increment formation may vary, thus causing difficulty in the interpretation of age from otolith microstructure, only a few studies have provided validation of the frequency of formation of increments (Beamish and McFarlane 1983). Indeed, the results of many studies carry the explicit assumption that the otoliths increments were formed daily (Campana and Neilson 1985). Otolith formation starts with a primordium, which is generally the first calcified tissue in the embryo (Dunkelberger *et al*., 1980). The nucleus is formed when the first discontinuous unit (Dunkelberger *et al.,* 1980) is laid down which corresponds to hatching, first feeding, or start of activity (Brothers & McFarland., 1981; Morales-Nin, 1992), although some species with long embryonic periods may start forming increments before hatching (Morales-Nin, 2000). Aside from the importance of counting daily increments for age and growth rate calculations, otolith microstructure examination may be also used to determine life history transitions, ambient temperature, trophic status, stock identification and taxonomic identification (Campana and Neilson 1985). Checks, or discontinuities (i.e. growth interruptions), which are characteristic of most otolith growth sequences, may record periods of perturbation or stress to the fish (Campana and Neilson 1985). Non-periodic check formation is generally associated with periods of stress or sexual maturity (Campana 1983), while periodic series may be linked to the lunar cycle (Pannella 1980).

The micro-chemical composition analysis has added another dimension to otolith studies (Green *et al*., 2009). Otolith composition is relatively pure compared to must biological and mineralogical structures, being dominated by calcium carbonate in a non-collageneous organic matrix. A total of 31 elements have been detected in otoliths to date, being the elemental composition dominated by the major elements calcium carbonate, oxygen and carbon wich

make up the calcium carbonate ($CaCO_3$) matrix. (Campana, 1999) other elements (e.g. Sr, Ba, Mn, Mg) occur in the otoliths structure, but at lower (<100 ppm) or even trace (<1000) levels of detection (Campana, 1999). The current interest in the chemistry of otoliths is driven by the environmental chronological capabilities of these structures rather that any unique chemical properties. Nonetheless, most of works realized with otoliths are possible only due to some particular characteristics of these structures; which make the excellent natural markers of the fish habitat and valuable tools for studies of fish life history and movements (Campana & Thorrold, 2001). Two key properties of the otolith underlie the use of the otolith elemental composition as a natural marker (1): unlike bone, the otolith is metabolic inert; therefore, newly deposited material is neither resorbed nor reworked after deposition (Campana & Neilson, 1985); and (2) trace element uptake onto growing otolith reflects the physical and chemical environment (Fowler *et al*, 1995), albeit with significant physiological regulation (Campana, 2005). Isotopic ratios of elements such as carbon and oxygen are similarly influenced by environmental availability and temperature. Such environmental responses, recorded permanently in the otolith, imply that the otolith concentration of selected elements and isotopes (the "elemental fingerprint") can be used as a biological tag to: examining the connectivity of coastal and estuarine fish populations (Secor *et al.,* 1995; Campana, 1999; Swearer *et al.*, 2003; Elsdon & Gillanders, 2004; Correia *et al.,* 2012), discriminating between fish stocks (Campana *et al*., 1994, 2000; Correia *et al*., 2011); tracking fish migration patterns (e.g. Gillanders & Kingsford, 1996; Secor *et al*., 2002; Hobbs *et al.,* 2005; Elsdon & Gillanders, 2006); quantifying natal homing in migratory fishes (Thorrold *et al*., 2001); identifying juvenile nursery areas (Gillanders & Kingsford, 2000; Forrester & Swearer, 2002; Secor *et al*., 2002; Correia *et al*., 2014) and assessing larval retention in reef fish (Swearer *et al.,* 1999). The real strength of natural tags is identifying natal origins, which distinguishes this method from other indirect approaches to measuring connectivity, including coupled biophysical modeling and population genetics (Thorrold *et al*., 2007).

The successful use of geochemical signatures to assess the levels of connectivity among local populations depends on the existence of substantial variation in the elemental composition of those tags among locations of interest (Thorrold *et al*., 2002). Specific elements and isotopes incorporated into growing surface of the fish otolith reflect the physical and chemical characteristics of the ambient water, although not necessarily water masses often produce otoliths of different elemental composition, the otolith elemental composition ("elemental fingerprint") serve as an environmentally induced tag of group of fish. These tag tend to be physically stable, reproducible, and different among stocks, but are not necessarily stable over long periods. Thus, they do not serve as a proxy for genetic identity. However

fingerprint is very stable over the short term, making it valuable as a seasonally stable biological tracer of predefined group of fish and because the layers of aragonite are deposited sequentially in otoliths through the fish lifetime, we can analyze the otolith signatures corresponding to a particular life cycle event (e.g. natal origin: otolith core analyses; moment prior to capture – otolith edge analysis) or through the entire fish life (e.g. whole otolith analyses).

1.4 Main objectives

1) Use the otolith microstructure, through LM and SEM studies, as a proxy to track important ontogenic events (such as hatching, first feeding, settlement and age at coastal recruitment) that take place during the *L. pholis* life cycle using wild individuals and controlled laboratory reared specimens;

2) Validate the primary incremental deposition in otoliths, through the use of fluorescent markers (such as, tetracycline and alizarin), in order to estimate age and the duration of the early developmental life cycles stages of individuals;

3) Examine the spatial variation of the microchemistry of otoliths, namely the isotopic and elemental signatures obtained through ICP-MS-LA and IRMS analyses, in *L. pholis* embryos and larvae collected in different geographic regions in order to construct an atlas of natal signatures;

4) Assess the natal origin of settlers/recruits in order to estimate the contribution each spawning/nursery area to the coastal recruitment;

5) Link the otolith fingerprinting data obtained from this rocky intertidal fish to the oceanographic knowledge of the Portuguese currents to address connectivity issues and to understanding the oceanic dispersal processes of marine organisms, in order to support the management of the coastal marine biodiversity.

The resulting improvement of the state of the art of *Lipophrys pholis* biology could be important in the future once could easily be used as a fish model to understand connectivity of marine populations, to monitor dispersal in planktonic fish larvae and to study fish recruitment processes.

1.5 Structure of the thesis

This PhD thesis is organized into five chapters. In chapter one, we present the state of the art, showing the accumulated knowledge of the *Lipophrys pholis* biology before the beginning of this thesis. The main objectives of this work is also included in this chapter. The middle chapters (2 to 4) are composed of journal articles already published (4), or accepted (1) or in preparation (1). In all manuscripts I am the principal author and it represent the main body of this doctoral thesis. Since in these sections each sub-chapter represents a paper, they include an introduction, material and methods, results, discussion, acknowledgments and references. In the main body of the thesis, the journal articles have been grouped based on the main subject addressed in each paper, although some subjects will inevitably overlap. Chapter two basically concerns the Ontogenetic development of *L. pholis* and validation of daily increment**.** Chapter three focuses on the main aspects of the early life history, age, growth and sex of *Lipophrys pholis*. In chapter four, I study otolith microchemistry to attempt to identify some L. *pholis* movements. Chapter five presents the final discussion and conclusions of the thesis, including some new questions left open and giving suggestions for future work on *L. pholis* biology. Finally the references cited in chapters one and five are presented.

CHAPTER 2

Ontogenetic development of *L. pholis* and validation of daily increment

2.1. Ontogenetic development of the sagittal otoliths of *L. pholis* during embryonic, larval and settlement stages.

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Abstract

Eggs and settlers of *Lipophrys pholis* were collected during the low-tides from a northern Portuguese rocky beach in May 2013. The eggs were reared under controlled-laboratory conditions until the larval stage, while settlers were immediately sacrificed. Sagittae were viewed by scanning electron microscopy to assess the occurrence of differentmicroestructural checks during embryonic, larval and settlement stages. Otoliths recorded several micro-increments (8-10) before hatching in late embryo phases. In larval otoliths a visible hatching check was observed and micro-increments were deposited on a daily basis. Early settlers presented in the otolith edge two types of settlement marksb. These findings are important to prevent a misidentification of some life history events and/or to avoid overestimation of individual age from otolith studies.

Key-words: blenniidae, sagittae, microstructure.

Introduction

The shanny *Lipophrys pholis* is a multiple spawning intertidal species (Qasim 1957) with a protracted breeding season (Ferreira et al 2012) common in northeastern Atlantic (Zander 1986). In Portugal the reproductive season occurs from October/November to May (Faria et al 1996), but in Great Britain it takes place earlier from Marc/April to August (Qasim 1957). During the breeding period males exhibit a typical dark coloration pattern and establish territories in crevices or spaces under stones where spawning takes place (Qasim 1957; Almada et al 1990). The duration of the embryonic period is related with the incubation temperature, ranging from 16 days at 17.0ºC (Faria et al 2002) to 43 days at 11.5-15.0ºC and 61 days at 9.5- 14.0 ºC (Qasim 1957).

During the ontogenetic development of *L. pholis* embryos, four different eggs stages were recorded (A, B, C and D) with the otolith being formed during stage C (Faria et al 2002). The newly-hatched larvae measure between 4.4 mm and 5.4 mm in total leght (Hefford 1910; Ford 1922; Faria et al 2002). External feeding behavior starts one day after hatching (Faria et al 2002). The otolith microstructure seems to be species specific and could record several ontogenetic transitions during the first developmental stages of fish, such as hatching, yolk-sac absorption, first feeding, settlement and metamorphosis (Wright et al 2002). For *L. pholis* periodicity of daily growth increment formation was recently validated in early juveniles (Carvalho et al 2014). However, at present, the ontogenetic development of otoliths is poorly known for this species. The present work aims to describe the microstructure of sagittae of *L. pholis* during its early ontogenetic stages through the use of scanning electron microscopy.

Material and methods

Egg-masses of *L. pholis* were collected in a northern Portuguese rocky beach (Póvoa do Varzim: 41°23'47.79"N 8°46'45.48"W) during March of 2013. After visual identification of *L. pholis* nests guarded by a male, each egg-mass (only stages C and D, Faria et al 2002) were placed into a labelled individual Eppendorf with cold seawater and transported to laboratory. In the laboratory, eggs were keep in aquaria with aeration and illuminated 85 with fluorescent light (18 W) under controlled conditions of photoperiod (12L:12D), salinity (36 psu) and temperature (16ºC). After hatching larvae were maintained under the same abiotic conditions and feed twice a day with *Artemia sp*., nauplii and rotifers. Larvae were sampled, almost on a daily basis, during three weeks, measured (0.1 mm) and stored in ethanol 70%. Since all larvae died before reaching the settlement stage, (the oldest survival larvae was 23 days old), wild settlers (20.0 ± 1.6 mm total length, TL) were collected by hand nets during low-tides in

rocky pools in the same beach in May 2013. Fish were kept in seawater cooled with ice and rapidly transported to the laboratory. After being anesthetized with lethal dose of 2-phenoxyethanol the specimens were preserved in ethanol (70%) for to further analysis. Larval and settler´s total length (TL) was corrected for shrinkage (10%) (Table1).

Table 1 Ontogenetic stage, number of individuals, total length and age of *L. pholis*.

Ontogenetic Stages	*n*	Total lenght (mm) Range (mean ± sd)	Days after hatching
Embryos (C and D)	5 + 5	1.7 -1.8 (1.8± 0.7)	-
Larvae	38	3.8 – 6.0 (4.7± 0.8)	0 - 23
Settlers	10	17.0- 21.0 (20.0± 1.6)	57 - 94

Sagittal otoliths were extracted from the otic cavity of embryos, larvae and settlers using a fine-tipped tungsten dissecting pin with the help of a stereomicroscope (Meiji, EMZ-13TRX) at magnifications between 15 and 60 X. Sagittae were cleaned of adherent tissues with ultrapure water (Milli-Q-Water), transferred using a superfine synthetic paintbrush to SEM specimen mounts (Pelco, Pin Stubs) and mounted with the sulcus acusticus down using Araldite glue (Standard, CEYS - huntsmanllc). Sagittal were manually grounded in the sagittal plane with silicon carbide paper (Hermes, 1200-4000) and polished with alumina paste (Struers, AP Paste) to expose the core. During this procedure frequent visual checks were made on the cores, viewed as dark spots, under a metallographic microscope (Meiji, ML7100) at 40X magnification. Thereafter otoliths were etched with hydrochloric acid (HCl) 0.5 M during 30 seg, vacuum-coated with gold and viewed under scanning electron microscope (SEM) (FEI Quanta 400FEG ESEM **/** EDAX Genesis X4M) at 15 kV at magnifications between 700X and 3500X. The diameter (OD), radius (OR) and increment width (IW) of sagittae were measured (µm) in their longest axis using a free software program (Olympus, Measure IT). The distance from the core to the first ring along the posterior axis was also measured and any distinct rings or changes in ring pattern were recorded. Settlement marks were visually identified through optical density transitions and abrupt change in the increment width of micro-increment (McCormick 1994). Linear regressions between otolith measurements (OD and OR) and TL of larvae and settlers were performed. The relation between TL and age (post-hatching days) for larvae and settlers was also analyzed. The daily otolith grow rate (OGR) was calculated by using the otolith radius from hatching check to the edge divided by the post-hatching days.. The deposition rate of micro-increments in reared larvae was evaluated using Student´s *t*-test (real post-hatching age vs number of post-hatching otolith micro-increments). Data were

presented a mean values ± standard deviations. A level of significance (α) was 0.05. Statistical analyses were performed using SigmaPlot 11 (Systat software Ianc.)

Results

The sagittae of the embryos/larvae were spherical in shape, convex on their distal side and somewhat concave proximally (Fig. 1a, b). For settlers, the sagittae were cuneiform to oval, with sinuate to lobate margins, and the longest and shortest axes of the otoliths were along the anterior-posterior and proximal-distal directions, respectively (Fig. 1h). The core, is located in the central region of the otoliths, showing the primordium as visible deep hole in the centre surrounded by several micro-increments (8 to 10 rings), until what is presumed to be hatching check, (HC) (Fig. 1d, f, h). The core presented an average diameter value of 14 ± 2 μm. The hatch mark was determined based on new born larvae (0 days-old) with a OR of 28 μm (SD=3.54, N=38). For the larvae the number of micro-increment was deposited in a daily basis (*t*-test: *t*=1.245; d.f. = 38; *P*<0·005). Afterwards, micro-increments are clearly visible and the settlement mark (SM) was located in the otolith's pheripheral zone of settlers (Fig. 1h). Two settlement mark types *Ia* was characterized by a sharp decrease in increment width across the settlement mark completed within a few increments; and the second one was a multi-increment transition mark, I*b.*

The OR (OD) ranged from 17 a 25 μm (30 to 49 μm) and 27 to 42 μm (48 to 77 μm) for embryos and larvae, respectively. A weak, but positive correlation was found between the otolith measurements (OR or OD) and the larvae length (TL) (Fig. 2a). For the settlers, the overall OR and OD ranged from 186 to 260 μm and from 361 to 478 μm, respectively. A moderately positive correlation was also observed between the otolith sizes and the fish length for settlers (Fig. 2b). The relationship between the TL and age was weak for larvae (TL = 0.08 AGE + 4.28, R^2 = 0.20, n = 38, P<0.05,) and moderate for settlers (TL = 0.11 AGE + 10.78, R^2 = 0.53, n = 10, P<0.05). OGR showed a negative linear relationship with age for both larvae (Fig. 3a) and settlers (Fig. 3b). Furthermore, the experimental data obtained from larvae showed a fast decrease in OGR during the first five days, but afterwards the otolith growth rate remained more or less constant, with a slower rate, reaching its minimum (around 0.5 lm/day) at day 14. The overall mean OGR for settlers was twice as higher as for larvae.

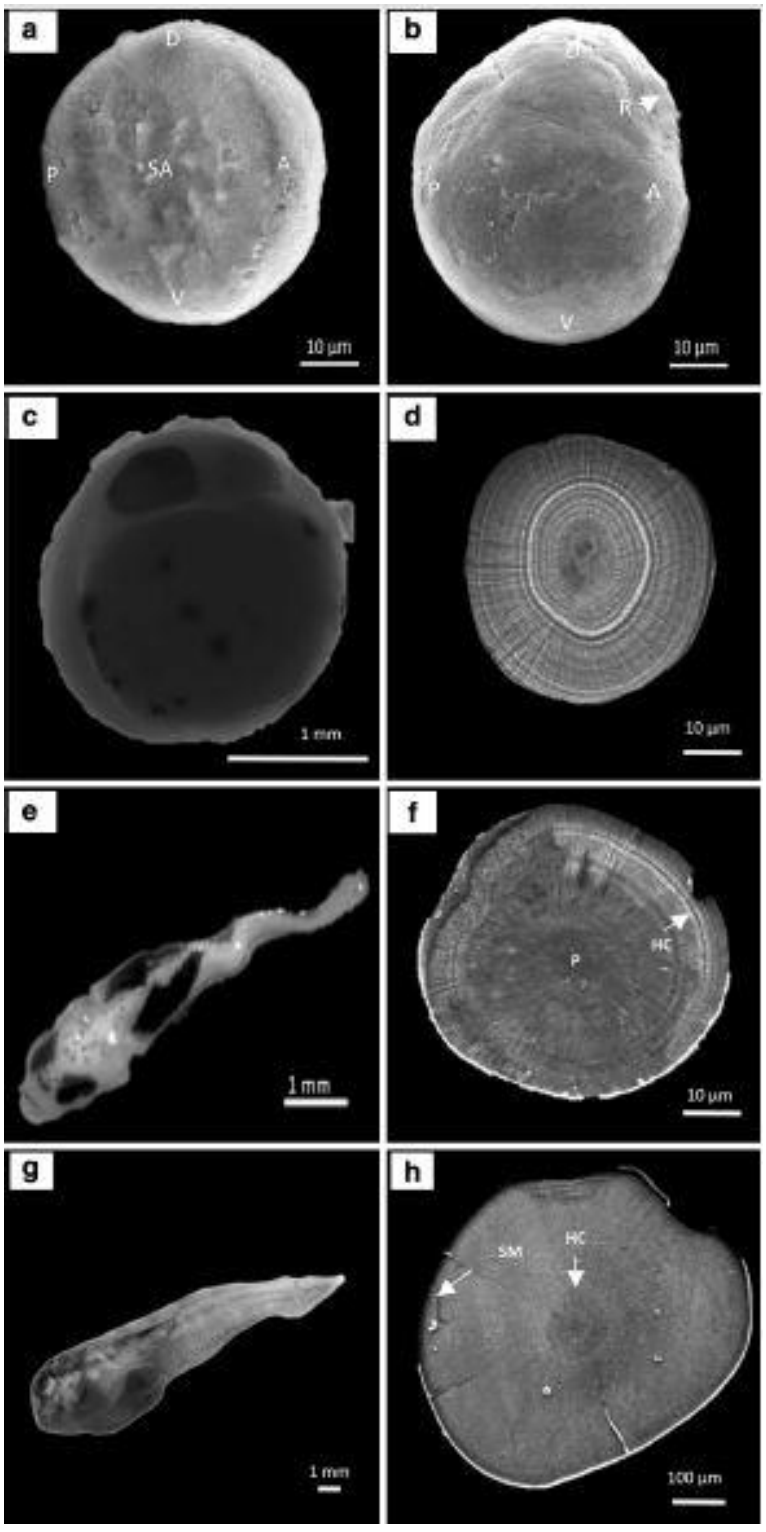

Fig. 1 Scanning electron microscope (SEM) images of the external features of the sagittae from 2-day-old larvae of *Lipophrys pholis* (4.7 mm total length), TL showing the medial side of the right sagitta (**a**) and the lateral side of the left sagitta (**b**). Photo of an *L.*pholis embryo in D stage (**c**) and SEM image of its sagitta (**d**) Photo of an 8-day-old larvae of L. pholis (5.3mm TL) (**e**) and an SEM image of its sagitta (**f**). Photo of an 60-day-old settler (16.0 mmTL) (**g**) and SEM image of its sagitta *A* Anterior ,*P* posterior, *D* Dorsal, *V* Ventral, *SA* Sulcus Acusticus, *R* Rostrum, *HC* Hatching Check, *P* Primordium and *SM* Settlement Mark

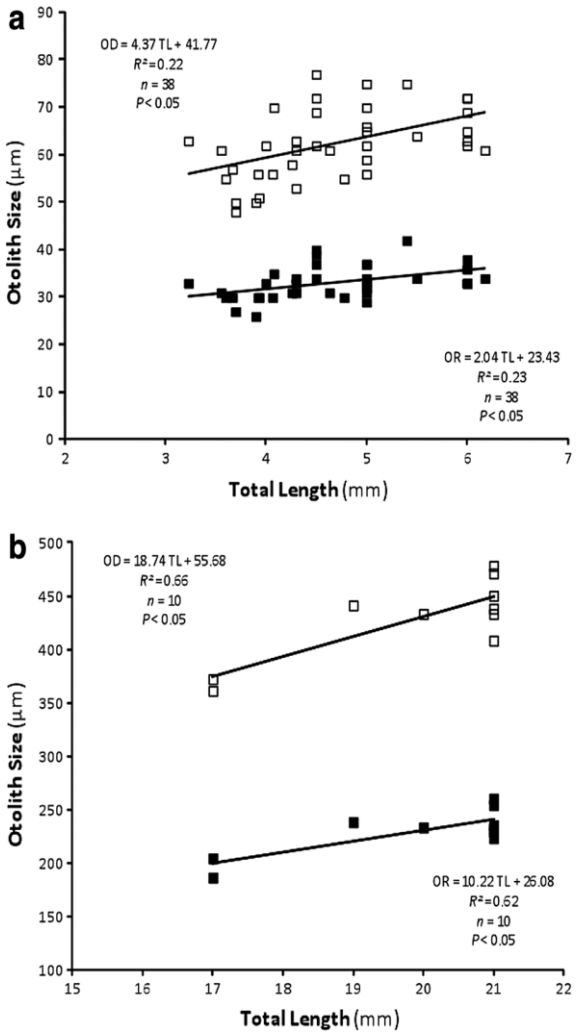

Fig. 2 Relationship between otolith size and fish length for larvae(a) and settlers (b) o*f Lipophrys pholis*. OD Otolith diameter (open squares), OR otolith radius (solid squares), TL total length

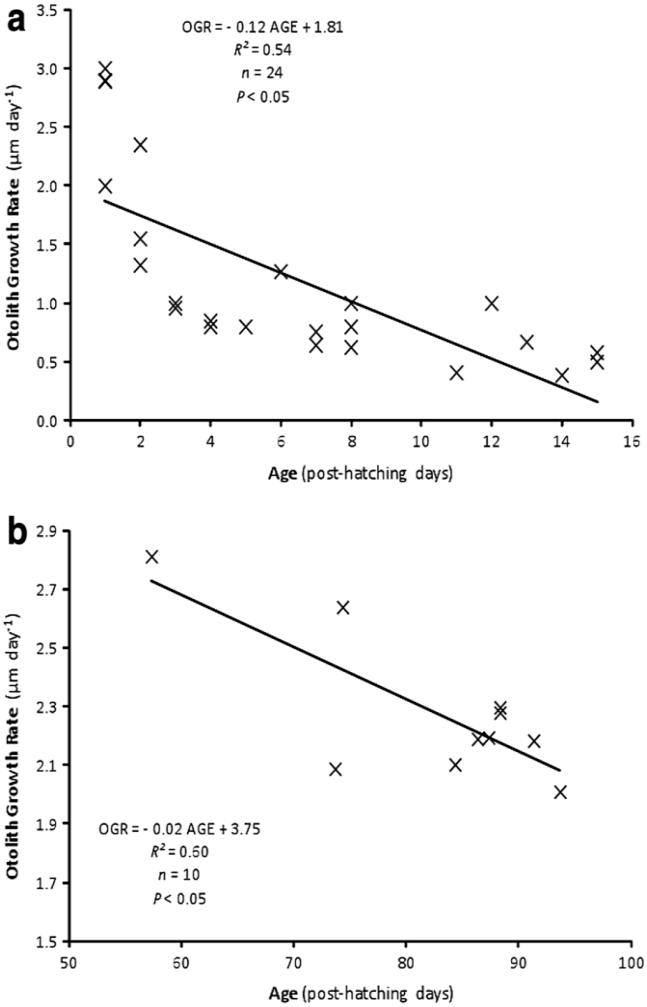

Fig. 3 Relationship between otolith growth rate (OGR) and age for larvae (a) and settlers (b) of *Lipophrys pholis*

Discussion

Sagittae are the largest otoliths and therefore easiest to handle and to examine (Campana and Neilson 1985). A few micro-increments were already visible in the center of the otolith before the hatching check and were considered to be formed prior to birth, i.e. during the embryonic development. Similar results were described for other species, such as *Perca fluviatilis* (Kristensen et al 2008), *Gobiesox marmoratus, Sicyases sanguineus* (Contreras et al 2013) and *Salmo trutta* (Dodson et al 2013). The formation of increments in otoliths inside the hatch check is probably related to ontogenetic developmental events, such as vascularization, eye pigmentation or development of other structures (Geffen 1983), and its frequency seems to be associated to fish with long incubation periods (Moyano et al 2012).

Other important life history events usually identifiable in fish otoliths are the larval hatching and the first external feeding (Campana and Neilson 1985).The otolith hatching check was clearly visible, but the first feeding check, which represents the period of final yolk absorption and onset of the exogenous feeding, was not observed. This yolk-sac absorption check was not distinguished in sagittae probably because of the short duration of the yolk-sac absorption in *Lipophrys pholis*, since the first feeding is described to occur one day after hatching (Faria et al 2002). Settlement is also another ecological and transitional event that is frequently evident in the otoliths, althoutgh recorded in different ways (Green et al 2009). In this study a settlement mark *type Ia* and *Ib* was identified in the peripheral region of the otoliths of settlers. This kind of settlement mark has been widely recorded in individuals belonging to Gobiidae, Gobiesocidae and Blenniidae families (Beldade et al 2007). The overall otolith daily growth rate for *L. pholis* larvae and settlers was 0.99±0.63 μm/day and 2.28± 0.25μm/day, respectively. These results showed that otolith growth rate was higher in settlers comparatively to larvae. However, these results should be analyzed carefully, since larvae were reared under laboratory conditions and probably they were in sub-optimal growing conditions which frequently induce fish stress and affect growth rate (Lambert 2003). Furthermore, the OGR observed in settlers are within the values obtained for *L. pholis* recruits (≤ 30 mm) (2.25 ± 0.50 μm/day to 3.03 ± 0.50 μm/day) captured across the Portuguese coast (unpublished data).

In this study, OR and OD showed a weak/moderate relationship with fish length of larvae and settlers. TL and age post-hatching was also weakly related for larval specimens. A similar weak relationshio between otolith and fish size at emergence has been described for *salmo salar* (Metcalfe 1992) and for *Salmo trutta* (*see* Titus and Mosegaard 1991). The size-selective mortaliy after hatching can lead to an artificial restriction of size ranges of young fishe that may influence the ability of regressions analysis to detect significant relationships between otolith and fish size (Meekan et al 1998). The basic assumption is that the main factor responsible for

growth of the otolith is somatic growth (Campana and Neilson 1985). However, other variables such as temperature and feeding regimes may increase the risk of uncoupling the relationship between otolith growth rate and fish growth rate (Folkvord et al 2004; Fey et al 2006). OGR showed also a negative linear relationship with age suggesting that younger individuals grow faster during their early stages was already expected and its currently explained by the available numerical growth curves (Katsanevakis 2006). The microstructure of otoliths is particularly useful to reveal ontogenetic or environmental events that can be used to infer pelagic larval durations, to reconstruct settlement patterns and to infer growth models (Campana and Neilson 1985). However, the interpretation of the microstructural growth of sagittae in *L. pholis* should be look with careful since it can lead to a misidentification of some life history events and/or cause overestimation of individual age.

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2.2. Validation of otolith daily increment in early juveniles of shanny *L. pholis*

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Abstract

To assess the periodicity of micro-increment formation in otoliths of *Lipophrys pholis*, 90 early juveniles were immersed in alizarin red S or tetracycline hydrochloride for 24 h and sacrificed after 10, 20 and 30 days. The number of micro-increments viewed under light microscopy was significantly related to the duration of the experimental period, and the slopes of the linear regressions were not significantly different from 1. This study indicates that micro-increments in sagittae were deposited daily and can be used as reliable sources of age information for *L. pholis*.

Key words: age validation; Blenniidae; fluorescent dyes; micro-increments; sagitta.

Introduction

The shanny *Lipophrys pholis* (L. 1758) is one of the most abundant fishes in the north-east Atlantic Ocean, found from Mauritania to Norway, including the Azores and Madeira Islands, and also in the Mediterranean Sea (Zander, 1986). *Lipophrys pholis* is an intertidal fish found on the rocky shores of Portuguese coast, but its geographical distribution extends to higher latitudes than other European blenniids (Almada *et al.*, 2001).

In Great Britain, *L. pholis* breeds during spring and early summer (Milton, 1983), whereas in Portugal the breeding season occurs in the cooler months, from October-November to May (Faria *et al.*, 1996). According to captive experiments at 17° C, embryonic development lasts 16 days after fertilization (Faria *et al.*, 2002). After hatching, larvae disperse to coastal areas, and early juveniles return within 2–3 months in early winter (Faria *et al.*, 1996), apparently to a particular set of rock tide pools, suggesting a kind of homing behaviour (Jorge *et al.*, 2012). After metamorphosis and settlement, the juveniles show a typical benthic behaviour (Faria *et al.*, 2002).

Lipophrys pholis could easily be used as a fish model to understand connectivity of marine populations, to monitor dispersal in planktonic fish larvae and to study fish recruitment processes. Knowledge about its population structure, fish movement patterns and connectivity is, however, at present limited. More information is required about the temporal occurrence of some life-history events in *L. pholis*. Otoliths have been successfully used during the last decades to age fishes, but a necessary requirement prior to using otoliths for ageing purposes is to validate the temporal deposition of the growth increments (Campana, 2001).

The aim of this study was to use fluorescent dyes to validate the deposition rate of the micro-increments in the sagittae of *L. pholis* under laboratory-controlled rearing conditions.

Material and methods

Lipophrys pholis were collected with hand-nets from rock pools during low tides in March and July 2013 at a rocky beach in the north of Portugal (Cabo do Mundo, 41° 13′ N; 8° 42′ W). Ninety fish 16 to 39mm total length (*L*T) were transported to the laboratory in refrigerated and aerated seawater containers and kept in quarantine for 14 days prior to experiments. Fish were maintained in 250 l tanks, with a re-circulated water system under controlled conditions of photoperiod (12L : 12D), temperature (mean±s.d. 17·2±0·7° C) and salinity (mean±s.d. 36·3±0·8).

Forty-five individuals were placed in a 25 l aerated seawater aquarium containing alizarin red S (ARS; Sigma-Aldrich, A5533, 100 mg l−1; wwwsigmaaldrich.com/) and in tetracycline hydrochloride (TC; Sigma-Aldrich, T3383, 400 mg l^{-1}) for 24 h in the dark to prevent light degradation of the fluorescent chemical. TC was also tested at 100 and 200 mg l^{-1} in two preliminary trials, but no fluorescent mark was produced. It was assumed that there was no lag-time between exposure to the treatment and incorporation of the chemical into the otoliths. Bath immersions were buffered by adding potassium hydroxide solution (KOH, ≥ 85%) to adjust pH (8. 02). After the bath, fish were randomly selected and placed in three replicate aquaria (*i.e*. 15 individuals per aquarium). The 25 l seawater aquaria were set with a controlled

photoperiod (12D : 12L), water temperature (mean±s.d. 15·8±0·3° C), pH (mean±s.d. 8·35±0·05) and salinity (mean±s.d. 35·2±0·6). Water physicochemical conditions were monitored daily with a multi-parameter probe (YSI, 556 MPS; www.ysi.com). Fish were fed *ad libitum* with frozen shrimps *Parapenaeopsis stylifera* and fresh mussels *Mytilus edulis* until they were sacrificed at 10, 20 and 30 days after exposure. All fish were anaesthetized with a lethal dose of 2-phenoxyethanol, measured (*L*T, mm) and preserved in 70% ethanol. Individuals that died before the end of the experiments were not used. Sagittae were extracted from the otic cavities of each individual under a stereomicroscope (Meiji, EMZ-13TRX; www.meijitechno.com/emz.htm) at ×15 magnification, and all adherent organic tissues were removed with 70% ethanol. Left otoliths were fixed with the sulcus acusticus down on microscope glass slides using epoxy resin (Buehler, EpoThinX; www.buehler.com). Resin was allowed to dry for 24 h in a dark room. Otoliths were ground in the sagittal plane with silicon carbide papers (Hermes, 2500; www.hermes-schleifmittel.com). During this procedure, frequent checks were made using light microscopy (Olympus, CX41; www. olympus. com/) until the core was revealed. At the end, alumina paste (Struers, AP-Paste; www.struers.com) was used for the final polishing of otoliths. The detection of the fluorescent dyes in otoliths was carried out through an ultraviolet (UV) microscope (Leica, DM6000B) using appropriate filters (Leica N2.1 and D, respectively, for ARS and TC; www.leica-microsystems.com). Otoliths were viewed at ×200, ×400 and ×1000 magnifications both in UV and in light microscopy. Microphotographs were taken using a USB digital camera (Olympus, SC30) and otolith morphometric measurements were made using a free software programme (Olympus, MeasureIT; www.soft-imaging.net). The daily otolith growth rate was estimated by measuring the maximum radius between the fluorescent mark and the otolith edge and dividing by the time elapsed. To examine the increment periodicity, the numbers of micro-increments from the fluorescent marks to the edge of the otoliths were blind counted by three independent readers. A linear regression was used to assess the degree of correspondence between the number of micro-increments and post-treatment days.

ANCOVA was used to compare the slopes of the linear regressions (three replicates) within each experiment (ARS or TC). To determine whether micro-increment formation was daily, the numeric value of the slope of the overall linear regressions of each independent experiment was evaluated using a one-sample *t*-test (*H*0 =1). The dailyotolith growth rate between the two experiments was also compared using a *t*-test. A level of significance (α) of 0·05 was used and data are presented as means±s.d.

Results

ARS and TC appeared as distinct bright red and yellow rings, respectively, when viewed under UV light in the otoliths of all early juveniles of *L. pholis* [Fig. 1(a), (c)]. Both marks were also clearly visible when viewed under normal light microscopy [Fig. 1(b), (d)]. The mortality rate observed during the chemical exposure itself and subsequent growth period for ARS (40%) was higher than for TC (16%).

The micro-increments were clearly visible from the fluorescent mark to the otolith edge for all experiments. The c.v. of repeated counts was <10%. No significant differences were detected among slopes (replicates) within each experiment (ANCOVAs: $F_{2,21} = 1{\cdot}439$, $P > 0{\cdot}05$ and $F_{2,32} = 1{\cdot}256$, $P > 0{\cdot}05$ for ARS and TC, respectively). The linear regressions of the number of micro-increments against time in the experiments for the individuals marked with ARS ($n=27$, $r^2 = 0{\cdot}98$, $P < 0{\cdot}05$) and TC ($n=38$, $r^2 = 0{\cdot}96$, $P < 0{\cdot}05$) were similar (Fig. 2). For both experiments, one-sample *t*-tests revealed that the slopes of the regression lines were not significantly different from 1·0 ($t = -1{\cdot}741$; d.f. = 2, $P > 0{\cdot}05$ and $t = -1{\cdot}328$; d.f. = 2; $P > 0{\cdot}05$, for ARS and TC, respectively).

The otolith radius and diameter of all individuals ranged from 195 to 450 μm and from 370 to 795 μm. The daily growth rate during the experimental period was 1·20±0·37 μmday−1 and 1·26±0·41 $\mu m day^{-1}$ for ARS and TC, with no significant differences (*t*-test: $t = -0{\cdot}559$, d.f. = 63, $P > 0{\cdot}05$). The overall otolith daily growth rate was 1·24±0·39 $\mu m day^{-1}$. Otoliths are valuable chronological tools for obtaining important ecological data from fishes, but usually require validation of the deposition rate of the otolith increments (Campana, 2001). This can be done in a direct manner by marking fishes, through fluorochrome dyes or capture–recapture experiments, or by the indirect use of statistical techniques (Panfili *et al.*, 2002). Chemical tagging is one of the best available methods to validate the periodicity of otolith increment deposition and can be done through intraperitoneal injection, dietary intake or bath immersion (Lagardére *et al.*, 2000). Chemical tagging by bath immersion in fishes involves a compromise between chemical concentration, immersion period, salinity, mortality rate, growing conditions and retention time (Liu *et al.*, 2009). Moreover, it has the advantage of being a less time-consuming, inexpensive and low-stress technique and a better option for juvenile fishes (Geffen, 1992).

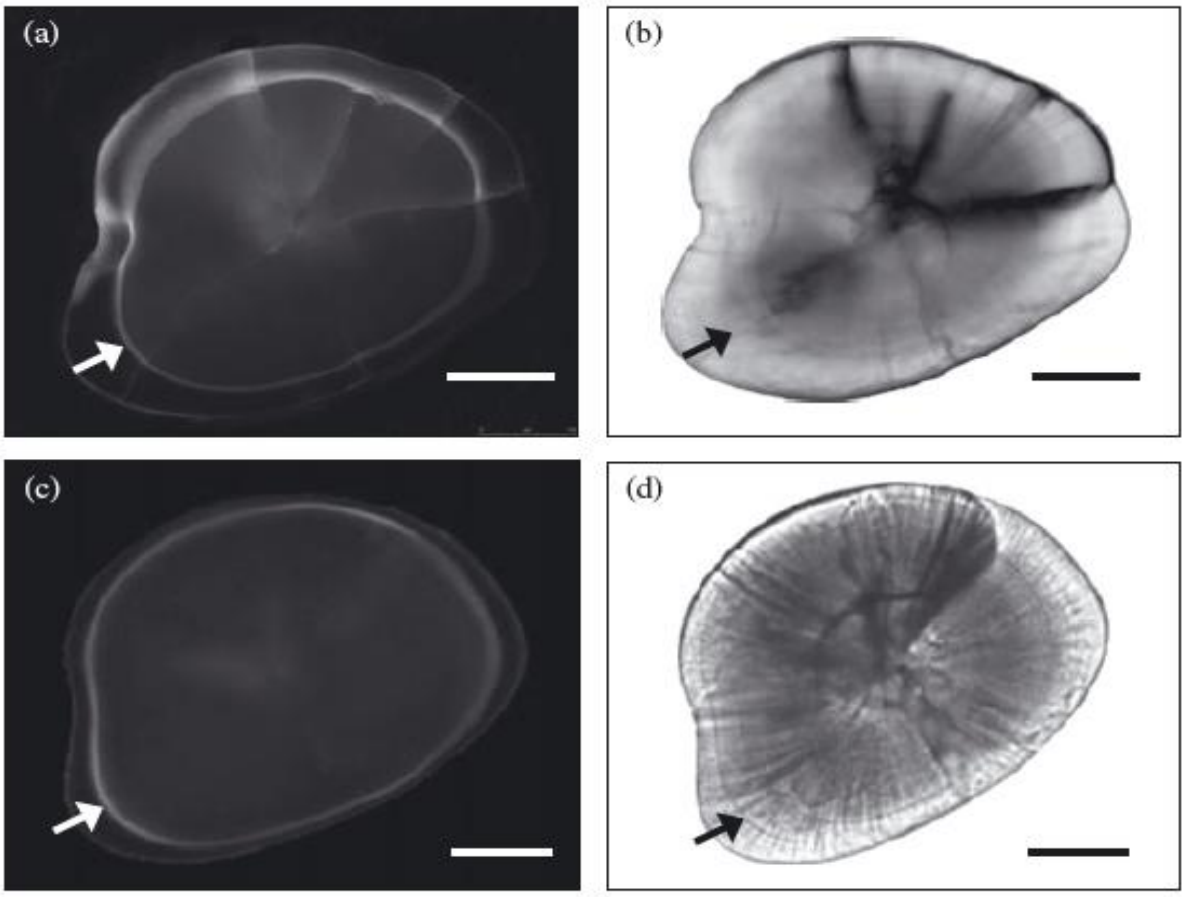

Fig. 1. Sagitta of *Lipophrys pholis* under (a), (c) UV and (b), (d) normal light marked with alizarin red S (total lenght, L_T=23 mm, sacrificed at 30 days) and tetracycline hidrocloride (L_T=22 mm, sacrificed at 10 days), respectively. ↓the location of the fluorescent marks. Bars =100 µm. Microphotographs taken at x 200 magnification

Alizarin and tetracycline are the most used fluorescent markers for otoliths (Panfili *et al.*, 2002). Although tetracycline seems to be the most commonly applied chemical (Hernaman *et al.*, 2000), it is also the most problematic because of its toxic effects (Geffen, 1992). Mortality rates are sometimes higher and the degree of incorporation in otoliths lower than for alizarin, or even calcein (Vigliola, 1997).

In this study, ARS and TC, used in standard concentrations and immersion bath times, gave good results in producing a visible fluorescent mark in the otoliths of all juveniles. It was necessary, however, to use a higher concentration of TC to mark the same number of individuals. The fact that TC chelates with the divalent cations dissolved in sea water could be the reason (Vigliola, 1997). In this study, mortality in ARS was greater than reported from similar ARS studies [Beckman & Schulz (1996): ARS, 100–200 mg l^{-1}, 3–14%; Ibáñez *et al.* (2013): Nile tilapia *Oreochromis niloticus* (L. 1758), ARS, 50–100 mg l^{-1}, 12%; Unfer & Pinter

(2013): sea trout *Salmo trutta* L. 1758, ARS, 150 mg l^{-1}, 13–31%]. The reported variation in mortality rates between studies is most commonly attributed to species, size of individuals and life stage of fishes, more than to the high concentration of dyes and the duration of the immersion period (Geffen, 1992; Vigliola, 1997; Liu *et al.*, 2009). Size of fish could be an explanation of the high mortality for ARS in this study. Fish used for ARS were significantly smaller than those used for TC (19·9±2·3mm *v*. 26·1±5·3 mm; *t*-test: $t=-5{\cdot}708$; d.f. = 63; $P<0{\cdot}001$) and are probably more vulnerable to the marking and handling procedure.

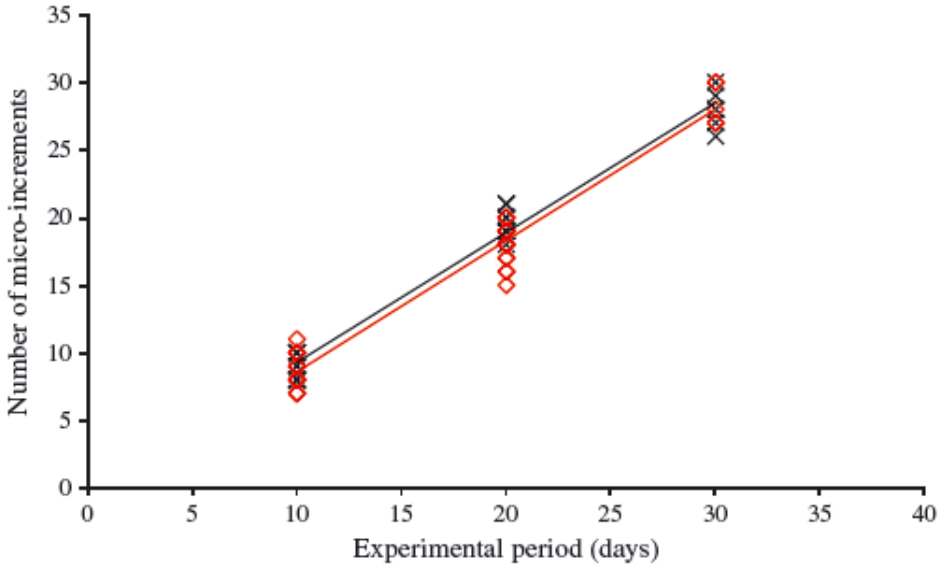

Fig. 2. Linear regressions between the micro-increments of marked otoliths (X, alizarin; ◊, tetracycline) and the number of days between marking and fish sacrifice. The curves were fitted by alizarin $y=0{\cdot}96x-0{\cdot}3$ and tetracycline $y=0{\cdot}97x-1{\cdot}1$

The data from this study indicate that under artificial conditions, the growth rate of the otoliths for early juveniles of *L. pholis* was *c*. 1·24 μmday $^{-1}$. Assuming that both marking methods had no effect on fish and otolith growth, similar values were reported for other perciforms (Shcherbich, 2005; Victor, 2007).

In blenniids, daily increment periodicity in otoliths has only been validated for molly miller *Scartella cristata* (L. 1758) (Grabowski, 2002) but was assumed to be daily in shanny *Lipophrys trigloides* (Valenciennes 1836) (Macpherson & Raventos, 2005).

Otolith daily increment deposition is, however, well known for other related taxa, such as gobiids (Hernaman *et al.*, 2000). In this study, a significant positive relationship was recorded between the number of micro-increments in otoliths and post-treatment days.

Furthermore, the slopes of the fitted linear regressions were not significantly different from 1. These data clearly indicate that the primary increments in sagittae of *L. pholis* were deposited daily. This study thus provides for the first time evidence that daily growth increments are reliable sources of age information for *L. pholis*. Moreover, the chronological micro-structural information recorded in otoliths can be used in the future to track life-history events of *L. pholis*, such as hatching date and age at coastal recruitment.

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CHAPTER 3

Aspects of the early life history, age, growth and sex of *Lipophrys pholis*

3.1. Pelagic larval duration, size at settlement and coastal recruitment of *L. pholis*.

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Abstract

To study some early life history traits of *Lipophrys pholis,* 110 recruits (TL ≤ 30 mm) were collected in April and May 2013 during the low tides periods in four rocky beaches along the west (Cabo do Mundo, Peniche and Vale do Homem) and south (Olhos de Água) Portuguese coasts. Pelagic larval duration, size at settlement and age at coastal recruitment were back-calculated from the microstructure of otoliths. Pelagic larval duration estimated from micro-increments counts until the settlement marks ranged from 57 to 73 days and showed a latitudinal reduction trend from north to south. This variable seems to be related in 30% with the regional seawater temperatures probably through the direct effect on the somatic growth. Settlement sizes (~ 19 mm) did not show any regional differences suggesting that this a more conservative character within species. The mean age at coastal recruitment varied between 69 and 93 day, but northern individuals were recruited at an older age. Back calculated spawning, hatching and settlement dates appear to be unrelated to the lunar cycle for *L. pholis*.

Keywords: Blennies, *sagittae*, micro-increments, early life history.

Introduction

For fish ecologists recruitment, in general, is defined as the number of individuals which survive from the eggs to a certain age or stage in their life history (Carr & Sims, 2006). In the present work recruitment is defined as the completion of settlement of pelagic larvae and return of the early juveniles to the adult spawning grounds. Early life history traits, such as duration of the planktonic larval stage and size-at-settlement, are closely linked to the fish recruitment success (Chambers & Leggett, 1987; McCormick, 1994; Radkte *et al*., 2001). These variables reflect the interaction of the individual´s developmental physiology with exogenous factors (e.g. food and temperature) (McCormick, 1994).

Temperature, in particular, causes variation in rates of fish development in the embryonic, larval and juvenile stages (Green & Fisher, 2004).

A decrease in the rate of ontogenetic development caused by a change in temperature usually results in a longer pelagic larval duration, increasing the exposure to the high risk pelagic larval environment (Atkinson, 1996). Moreover, through its effect on growth, temperature can influence the size of the organism at which ontogenetic transformations occur (Green & Fisher, 2004), which may determine the fish's subsequent growth schedules and survival (McCormick & Molony, 1995). Several studies have suggested that the pelagic larval duration seem to be a flexible early life history trait in both littoral and demersal fishes (McCormick, 1999; Sponaugle et al., 2006; Kendall et al., 2013). However studies on the conservative or flexible pattern of the size at settlement in fish are, at present, scarce (Juncker et al., 2006).

The shanny *Lipophrys pholis* (L. 1758) can be easily used as a fish model to understand connectivity of marine populations, to monitor dispersal in planktonic larvae and to study recruitment processes. It is an intertidal blenniid fish usually found in NE Atlantic and Mediterranean Sea shores (Zander, 1986; Almada et al., 2001). In Great Britain, *L. pholis* breeds during spring and early summer (Milton, 1983), while in Portugal it occurs in the cooler months, from October/November to May (Faria et al., 1996). At higher latitudes the reproductive season tends to start later and to end sooner, when conditions become favourable for larval dispersion and juvenile growth; while in low latitudes *L. pholis* has a protracted breeding and recruitment season (Conover, 1992). The reproductive season should end when the time available is not enough for late-born juveniles to grow and reach the minimal size to survive during the winter (Conover, 1992). In Portugal, the early juveniles can grow almost without interruption during the warmer months and those which recruit in early winter are able to reach the minimum size to be sexually mature within 1 year (Faria et al., 1996). During the breeding period the males establish territories in crevices and stones where

spawning takes place (Qasim, 1957; Dunne, 1977; Almada et al., 1990). The nests contain 3–8 batches of eggs from a single or multiple females deposited at different times during the course of a breeding season (Qasim, 1957). It is also known that L. pholis males are capable of multiple spawning episodes (Ferreira et al., 2011).

According to captive experiments embryonic development lasts 16 days at 17°C (Faria et al., 2002). After hatching the pelagic larvae disperse to the coastal area and individuals apparently return to a particular set of rock tide pools, 2–3 months later, in early winter to settle (Faria et al., 1996). Recent findings show that *L. pholis* adults can orient themselves toward their home pools, suggesting that homing abilities may begin with the onset of sexual maturation and not during a hypothetical imprinting phase during larval development, such as other marine species (Jorge et al., 2012). After metamorphosis and settlement, characterized by pronounced morphological and physiological changes, early juveniles (15–16 mm) show a typical behavior associated with a benthic mode of life (Qasim, 1957; Faria & Almada, 2001; Faria et al., 2002). Recruitment of fishes, 20 mm ceases 3 months after the end of the breeding season (Faria et al., 1996). Information regarding the temporal occurrence in the wild of these early life history events is nonexistent, because available data have been obtained through captive experiments.

This paper examines, for the first time, some life history traits of *L. pholis*, such as duration of the pelagic larval stage, size at settlement and age at coastal recruitment, inferred from the otolith microstructure of early recruits.

The effect of the lunar cycle on the timing of these early life history traits is also explored. This information jointly with the historical collection data from surface seawater temperatures form the basis of a discussion about the larval growth, settlement and juvenile recruitment mechanisms for this species.

Materials and methods

Biological sampling

One hundred and ten recruits (young juveniles ≤ 30 mm) were collected in April and May 2013 in four rocky beaches equally spaced (_300 km) along the Portuguese coast from north to south (Cabo do Mundo: 41813′N 8842′W; Peniche: 39826′N 9813′W; Vale do Homem: 37822′N 8849′W; and Olhos de Água: 37805′N 8811′W) (Table 1; Figure 1). Individuals were captured with handnets in rocky pools during the low-tide periods. For each site individuals were collected from three tide pools (replicates) spaced at about 50 m apart. Fish were kept in seawater cooled with ice, rapidly transported to the laboratory and killed with a lethal dose of 2- phenoxyethanol. All fishes were measured (total length: TL, 0.1 mm), distributed by size

classes (at intervals of 1 mm) and frozen (220°C) in Eppendorf tubes filled with seawater prior to further analysis.

Otolith microstructural analysis

Sagittal otoliths were carefully extracted from the otic cavity of fishes using a binocular microscope and cleaned of adherent tissues with ultrapure water (Milli-Q-Water). Left otoliths were mounted on microscope glass slides with the sulcus acusticus down using a drop of epoxy resin (Buehler, EpoThin). The otoliths were manually ground in the sagittal plane with silicon carbide paper (Hermes, 2500) and polished with alumina paste (Struers, AP Paste) to expose the core. Whole otoliths were photographed in a light microscope (Olympus, CX41) coupled to a USB digital camera (Olympus, SC30) at 200× and 400× magnifications. Images were acquired using a computer program (Olympus, AnalySIS getIT). If needed, successive series of microphotographs from each otolith were made to obtain a complete image of the otolith radius. The quality of the digital images was improved using a free software program (Paint.NET v3.5.10). The diameter and radius of otoliths were measured (mm) in their longest axis using a free software program (Olympus, MeasureIT). To back-calculate the spawning time, 16 days were added to the counted micro-increments, which corresponds to the number of days before hatching (i.e. embryonic period) (Faria et al., 2002). It was also assumed that the first micro-increment represents the hatching check as observed in other related species (Raventós & Macpherson, 2001). The micro-increments in sagittae are deposited on a daily basis in early juveniles of this species (Carvalho et al., 2014).

Pelagic larval duration was estimated by counting the daily rings from the hatch check until the settlement marks (McCormick, 1994). Settlement marks in the otoliths were visually identified using the optical density transitions and the abrupt change in increment width(s) (McCormick, 1994). The formation of the settlement marks was recently validated for this species and occur at the transition from the pelagic to the benthic environment in new settled fish (Carvalho et al., 2015). The number of micro- increments was blind counted by three independent readers and average values were used. Otoliths in which the coefficient of variation was higher than 10% were rejected. The averages of every 10 successive increment widths from the hatch check to the otolith edge were used for otolith growth-increment analysis.

Sea surface temperatures

Sea surface temperature varies on multiple temporal and spatial scales along the Portuguese coast (Lemos & Sansó, 2006). The mean daily sea surface temperature experienced by each fish during the pelagic larval duration was retrospectively estimated taking into account the date estimated from otolith microstructure (i.e. period of time from the hatching check until the settlement marks) overlapped with the available historical data of the sea surface temperature from the Portuguese coast. The sea surface temperature data were obtained from floating Datawell coastal buoys of the Instituto Hidrográfico da Marinha Portuguesa located nearest the L. pholis sampling points and anchored near the 100 m bathymetry (Leixões CSA92/D: WGS 84 – 41819′N 8859′W, depth: 83 m; Nazaré CSA88/1D: WGS 84 – 39833′N 9812′W, depth: 88 m ; Sines CSA83/1D: WGS 84 – 37855′N 8855.73′W, depth: 97 m; and Faro CSA82/D: WGS 84 – 36854′N 7853′W, depth: 93 m) (Figure 1)(Table1). It was also assumed that *L. pholis* larvae may be locally retained within the coastal environment since rocky intertidal fish larvae appear to be able to avoid off-shore dispersal (Marliave, 1986).

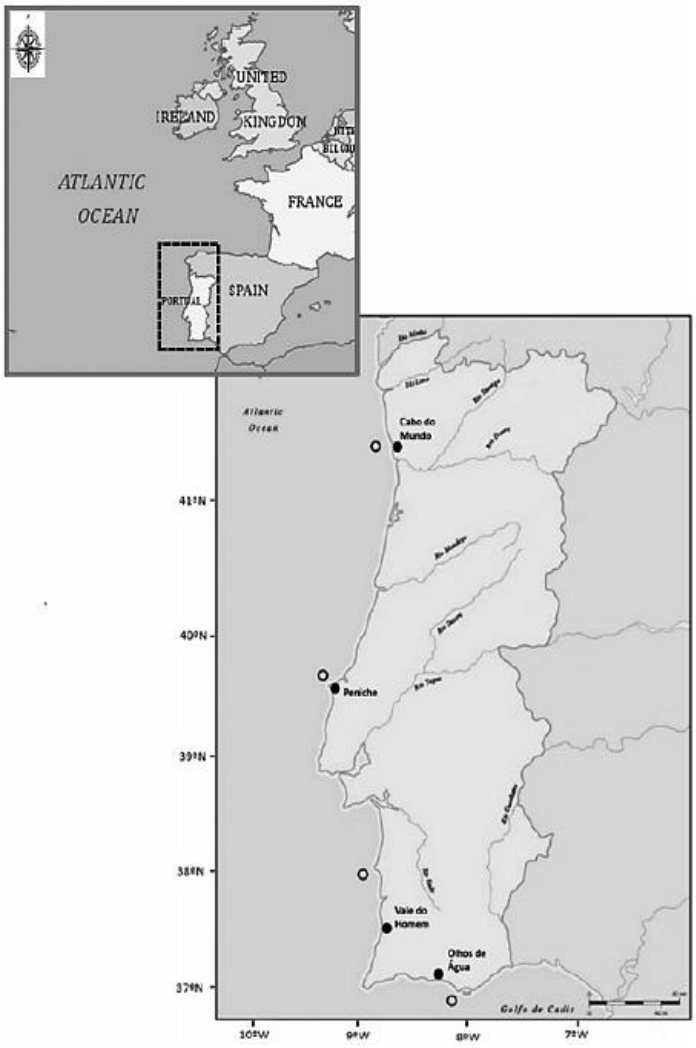

Fig. 1 – *Lipophrys pholis* sampling regions (black dots) and SST buoy positions (open dots) along the Portuguese coast

Table 1. Sampling location, collection date, fish length, spawning and hatching dates, size at settlement, age of recruits, pelagic larval duration, somatic growth rate, otolith growth rate and individual sea surface temperatures for *Lipophrys pholis* used in this study. Data were present as mean ± SE.

Sampling site	Collection date	n	Total length (mm)	Spawning date	Hatching date	Size-at-settlement (mm)	Age of recruits (days)	Pelagic larval duration (days)	Somatic growth rate (mm day^{-1})	Otolith growth rate (μm day^{-1})	Sea surface temperature (ºC)
Cabo do Mundo	02.05.13	33	16-30 (22.9 ± 0.6)	17.12.12 - 18.02.13	27.02.13 -14.04.13	18.6±0.2	109 ± 3	73 ± 1	0.219	2.25 ± 0.08	13.10 ± 0.05
Peniche	08.05.13	38	15-30 (22.1 ± 0.7)	03.01.12 - 31.03.13	11.03.13 - 07.05.13	18.9±0.2	93 ± 3	64 ± 1	0.248	2.35 ± 0.08	13.31 ± 0.05
Vale do Homem	27.04.13	17	15-30 (25.9 ± 1.3)	01.01.13 - 25.02.13	26.02.13 - 04.05.13	19.1±0.4	94 ± 4	57 ± 2	0.289	2.90 ± 0.15	14.08 ± 0.09
Olhos de Água	26.04.13	22	16-30 (23.8 ± 0.9)	05.01.13 - 19.03.13	17.02.13 - 07.05.13	19.1±0.3	85 ± 3	58 ± 3	0.313	3.03 ± 0.11	14.92 ± 0.07

Data analysis

All data were normally distributed (Shapiro–Wilk's test) with equal variance (Levene's test) after log10 transformation. One-way analysis of variance (ANOVA) was used to explore the mean differences in pelagic larval duration, size at settlement and age of the recruits between locations (factor), followed by a Tukey post hoc test, if needed. Linear regression analyses were used to examine the relationships between pelagic larval duration and sea surface temperature, and between otolith measurements (otolith diameter and radius) and total length (TL) of recruits. The somatic growth rates obtained from the slopes of the linear regressions between TL and age of the recruits for each site were used to retrospectively estimate the individual size at settlement. One-way analysis of co-variance (ANCOVA) was used to compare the regional somatic growth rates. The age of the recruits was used to back calculate the spawning, hatching and settlement dates (identified through the settlement marks) for each fish. The distribution of these activities over the lunar cycle was also estimated for each individual. The duration of lunar cycle was considered 29.53 days, the new moon was set as the first day of the lunar calendar and each lunar phase was encompassed by the day of each quarter phase +3 days (Sponaugle & Pinkard, 2004). Rayleigh circular statistics were used to test for the occurrence of nonrandom distribution of these activities through the moon cycle (Batschelet, 1981). All analyses were performed according to standard statistical procedures (Zar, 1996). A level of significance (a) of 0.05 was used. Data were presented as mean values ± standard error (SE).

Results

Micro-increments were clearly visible between the hatching check and the otolith edge. Two different otolith increment width profiles were observed along the radius of sagittae. A regular increase of the increment width was observed from the hatch check to the following 35 to 55 days (3.0–3.5 µm) for all individuals/sites. Afterwards there was a decrease of the increment width that reached the initial value (1.5 µm) at the otolith edge (110 to 120/130 days) for the northern individuals. For the southern individuals after this initial decrease until the 65 days, there was a steady increase in the increment width through the otolith radius with a final drop in the otolith edge. The visual analysis of the plot showed an abrupt increase of the width of increments that took place in the zone where the settlement occurred (40 to 55 days post - hatching) (Figure 2).

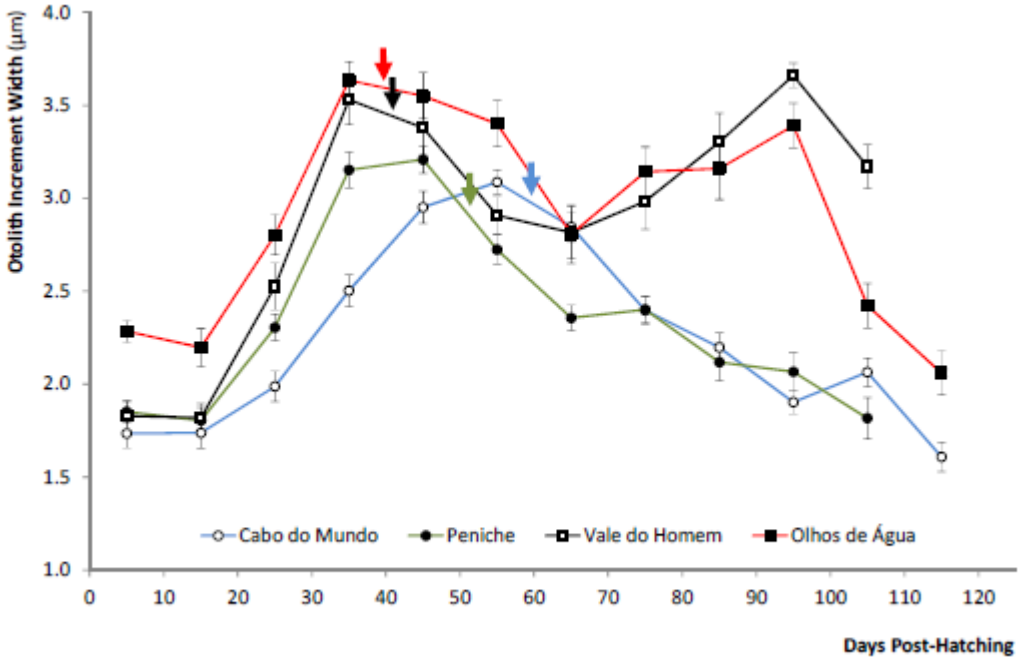

Fig. 2 – Profile of the micro-increments width (mean values ± standard errors) from the hatch check to the otolith edge. The individuals captured were grouped ccording to the sampling regions. The settlement mark formation was also identified (arrows).

The settlement marks appeared to be of two different types (Figure 3). A sharp decrease in increment width across the settlement mark completed within a few increments was categorized type Ia and a multi-increment transition mark was categorized type Ib. These two settlement marks were observed in 62% and 38% of the individuals, respectively. There were significant differences in the pelagic larval duration of *L. pholis* between sampling regions (ANOVA: $F_{3,106} = 61.999$, $P < 0.05$), except for the two southern locations (Tukey test, $P > 0.05$). There was however a general shortening of the pelagic larval duration from north to south (Cabo do Mundo: 73 ± 1 days; Peniche: 64 ± 1 days; Vale do Homem: 57 ± 2 days; Olhos de Água: 58 ± 3 days) (Table 1).

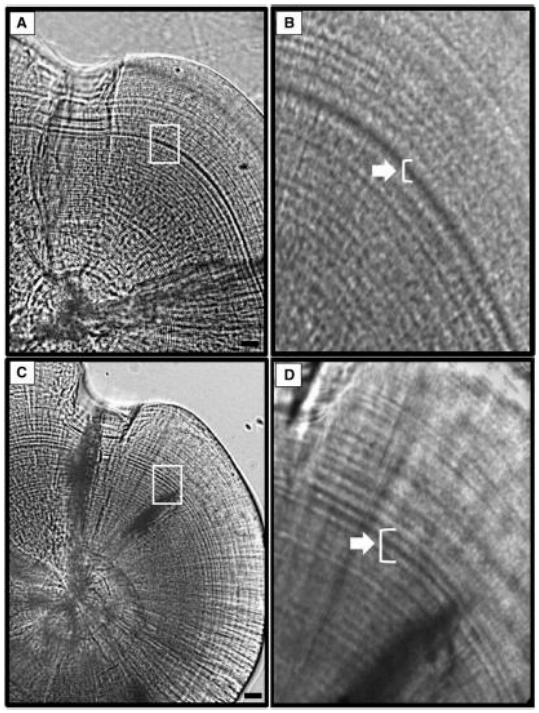

Fig. 3- Light microscope images of two otoliths of *Lipophrys pholis* individuals collected in Cabo do Mundo (A: TL= 21 mm; age= 84 days; C: TL= 23 mm; age= 100 days) showing in a zoomed selected area (white boxes) the type Ia (B) and Ib (D) settlement marks (white arrows). 200 x magnification. Scale bar = 10 μm.

The overall PLD was 64.4 ± 0.8 days (coefficient of variation of 12.4%). The variation of the pelagic larval duration could be explained in 30% by the sea surface temperature (Figure 4).

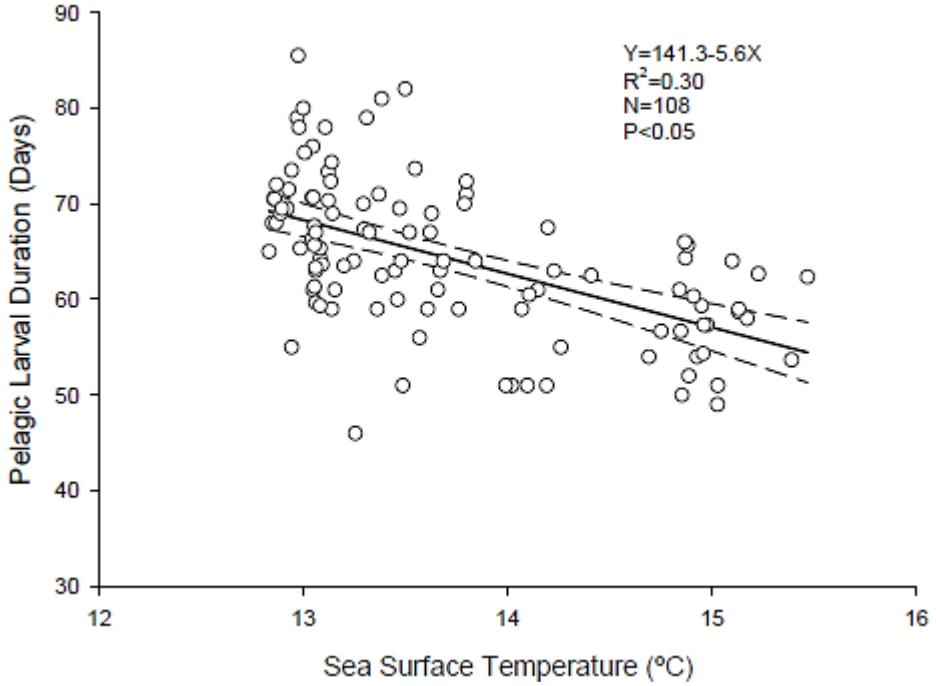

Fig. 4- Relationship between the pelagic larval duration and the average surface temperature experienced by each individual over the period between hatch and settlement. The dotted lines represent the 95% interval the linear regression

The overall otolith radius and diameter ranged from 138 to 323 mm and from 263 to 588 mm, respectively. A positive significant correlation was found between the otolith measurements and the fish length (otolith radius: Y= 9.56X + 19.38, R^2 = 0.82, N= 110, P < 0.05; otolith diameter: Y = 17.99X + 39.90, R^2 = 0.86, N = 110, P < 0.05). A significant positive relationship was also found between fish length and age of the recruits for all the sampling sites (Cabo do Mundo: Y = 0.22X + 2.60, R^2 = 0.83, N = 33, P < 0.05; Peniche: Y = 0.25X + 2.87, R^2 = 0.88, N = 38, P < 0.05; Vale do Homem: Y = 0.29X + 2.37, R^2 = 0.88, N = 17, P < 0.05; and Olhos de Água: Y = 0.31X + 2.16, R^2 = 0.85, N = 22, P < 0.05) (Figure 5). The size at settlement was estimated for each individual, but did not present any significant regional differences (ANOVA: $F_{3,103}$ = 2.595, P = 0.057) (Table 1). The average size at settlement was 18.9 ± 0.1 mm (coefficient of variation was 5.9%). All fishes settled between 18 and 20 mm long.

The age determination of the recruits from *L. pholis* captured at Cabo do Mundo, Peniche, Vale do Homem and Olhos de Água ranged from 57 to 119 days, 50 to 109 days, 45 to 100 days and 49 to 95 days, respectively. There were significant differences in the age of the recruits of *L. pholis* between sampling regions (ANOVA: $F_{3,106}$ = 12.769, P < 0.05), with the north location recording the older individuals (Tukey – test, P , 0.05) (Table 1). Significant differences exist between regional somatic growth rates (ANCOVA: $F_{3,105}$ = 3.35, P = 0.02), except for the two southern regions (ANCOVA: $F_{1,36}$ = 0.36, P = 0.55) (Table 1).

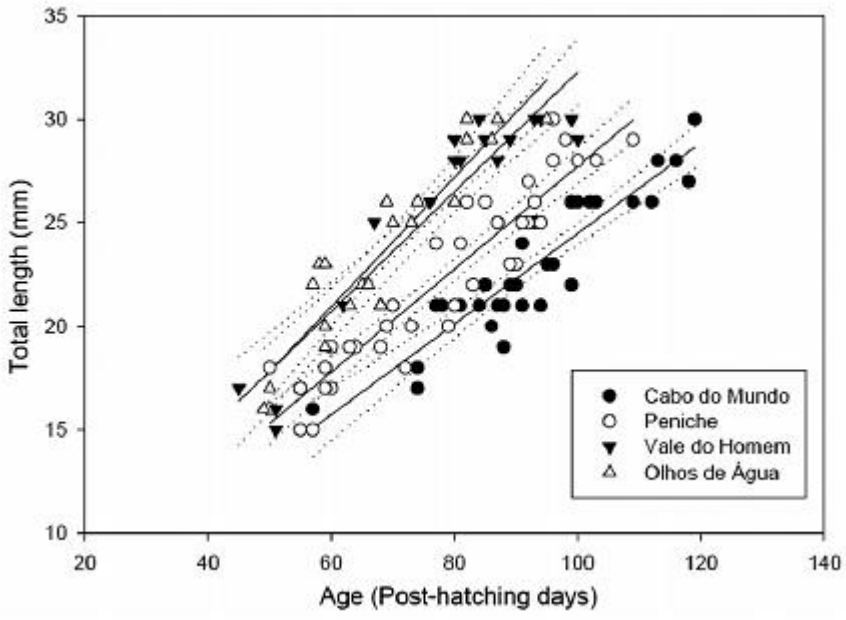

Fig. 5- Linear relationship between length and age at capture for the recruits for each sampling region. The dotted represent the 95% intervals for the linear regressions.

The observed frequency dates for the spawning, hatching and settlement of *L. pholis* (Table 1) showed that these biological events occurred during all phases of the lunar cycle, and none of these activities was related with a particular phase of the moon (Rayleigh Tests: $P > 0.05$).

Discussion

As expected, both otolith morphometric measures showed a good relationship with fish length (TL) (Searcy & Sponaugle, 2001). Furthermore, two settlement mark types were identified, Ia and Ib, both characterized by a sharp decrease in the increment width and completed within a few micro-increments (Wilson & McCormick, 1999). These settlement marks have already been identified among individuals belonging to Gobiidae, Gobiesocidae and Blenniidae families (Beldade et al., 2007), and occur in the peripheral region of the otoliths of *L. pholis* early settlers (Carvalho et al., 2015).

The present results indicate that the most common settlement mark for *L. pholis* was type Ia with 62% of frequency. Settlement mark types are known to vary among species, even within the same genus (Wilson & McCormick, 1999). In some cases (e.g. *Gobius xanthocephalus* or *Gobius paganellus*), such as in the present study, two subtypes of settlement marks could even be present within the same species (Beldade et al., 2007). Mark type Ia also seems be the most common settlement mark in more than 40 tropical species (Spounagle & Cowen, 1994), and appears also to occur in 68% of Mediterranean littoral fishes, including individuals belonging to *Lipophrys* genera, i.e. *L. adriaticus*, *L. canevae* and *L. trigloides* (Raventós & Macpherson, 2001). The structure of the settlement marks is species-specific, but dramatic reductions in increment width at settlement (i.e. individuals with type I settlement marks) are related to fish in which the timing of settlement differs slightly among individuals due to the influence of the environmental history on the developing larvae (Wilson & McCormick, 1999). It is plausible that these settlement marks reflect *L. pholis* individuals that settled successfully (type Ia) or individuals that settled in an unsuitable (or occupied) habitat and then moved before settling again (type Ib).

The estimate of the larval duration from otolith microstructure of *L. pholis* revealed a latitudinal pattern, i.e. a general shortening of the pelagic larval duration from north to south regions. It is well known that variation in pelagic larval duration may result from various environmental factors such as temperature, food availability and local current patterns (Jones, 1986; Lobel & Robinson, 1986; McCormick & Molony, 1995). In the present study the shorter pelagic larval duration occurred in the regions with warmer seawater temperatures (Vale do Homem and Olhos de Água); in contrast, the longest pelagic larval duration occurred in Cabo

do Mundo, the region with the coldest seawater. Furthermore, 30% of the variation in pelagic larval duration was explained by individual mean temperatures experienced by larvae calculated from local sea surface temperature. These results corroborate that temperature is a dominant influence on pelagic larval duration, which decreases exponentially with increasing temperatures across species and populations of marine fish (McCormick & Molony, 1995; Benoît et al., 2000; Green & Fisher, 2004). Results obtained for other tropical reef fish also reported that seawater temperature changes accounted for about 30% of the variation in larval growth (McCormick & Molony, 1995; Meekan et al., 2003; Sponaugle et al., 2006). Moreover, the effect of water temperature on larval growth leads to important environmental effects on the recruitment success (Spounagle, 2010).

Daily growth for a fish can be indirectly obtained by examining the width between successive increments in otoliths. The comparison of increment widths during a particular stage of life among individuals could provide a relative measure of somatic growth (Green et al., 2009). Otolith increment widths recorded in *L. pholis* are consistent with the values reported for other related fish families (Wilson & McCormick, 1999). Moreover, micro-increment widths measured in *L. pholis* otoliths showed a regional variation and sites with higher sea surface temperatures had higher otolith growth rates, which is particularly evident in the peripheral rings.

The effect of environmental factors in the daily growth pattern of otoliths during the fish's early life stages have been extensively studied using time-series data analysis (Maillet & Checkley, 1991; May & Jenkins, 1992; Searcy & Sponaugle, 2001), and it is generally accepted that there is a positive relationship between otolith growth rate and temperature (Campana & Neilson, 1985). Observations, both in the field and in the laboratory, have shown that microincrement width may change in response to temperature and diet, although the period of micro-increment deposition remained daily (Morales-Nin, 2000). Recently a field study showed that for fish larvae (*Sprattus sprattus*), otolith width increments could be closely related with in situ water daily temperatures (Baumann et al., 2006). These results are, as expected, coincident with the somatic growth rate which was positively related with temperature, and northern fish presented a slow somatic growth compared with the most southern individuals. The size at settlement of *L. pholis* appears to be, however, a conservative characteristic in this species. No significant differences were observed in the size at settlement for the sampling areas. Fish settled when around 19 mm long. These results are in agreement with regular field observations of the smallest fish found in tide pools which are on average 17.4 mm and behave like benthic juveniles (Faria et al., 2002). Furthermore, it suggests that fish need to reach a minimum size to begin the settlement process. This could

easily explain why southern fish settled earlier than northern individuals. If settlement is triggered by a minimum size, fish with lower growth rates should reach this minimum size later. In general the variation in size is less than variation in age at metamorphosis for marine fish and the required size may ameliorate competitive effects and reduce the risk of predation in the period immediately following metamorphosis and settlement (Chambers & Leggett, 1987). This agrees with the 'competent size' hypothesis which defends the idea that a flexible pelagic larval duration is needed to maximize competent size, because environmental conditions may change within a season and among years (Pastén et al., 2003). As *L. pholis* individuals need to reach a certain size to settle it can be hypothesized that in northern and colder waters the pelagic larval duration should be long and the settlement success therefore limited compared with further south. . It means that the survival to the larval stage driven by water temperature could be an explanatory factor of the overall geographic distribution of the species.

It is known that various life history events for fish species that inhabit temperate and higher latitudes are often synchronized with periodic changes according to the moon-related cycles (Takemura et al., 2010). In this study, the examination of spawning, hatching and settlement dates of *L. pholis* surviving individuals suggests that these biological events were apparently acyclic and continuous over the lunar cycle. Similar results were described for the spawning of *Ophioblennius steindachneri* (Robertson et al., 1990) and *Thalassonma bifasciatum* (Sponaugle & Pinkard, 2004; Sponaugle et al., 2006). Furthermore for *Sebastes inermis* the parturition dates were uniform within years over the lunar cycles in almost all settlement groups (Pastén et al., 2003). These results are consistent with previous reproductive studies for *L. pholis* which showed that it is an asynchronous spawner, with eggs being produced in several batches during the breeding season (Ferreira et al., 2012). It is possible that the asynchronous production of multiple batches function as a bet hedging strategy, allowing the eggs to be distributed among several males, thus reducing the risks of complete loss of progeny because of inadequate mate choice, environmental constraints and failure in larval recruitment, among other equally valid causes (Morrongiello et al., 2012). Regarding hatching and settlement events, both were also randomly distributed over the lunar cycle. Settlement patterns scattered throughout the lunar cycle have not been documented for any other species. For the gobidae *Coryphopterus glaucofraenum* for instance, settlement appears to occur in several large pulses associated with various lunar phases (Spounagle & Cowen, 1994). A plausible explanation can be supported by the fact that flexible pelagic larval duration in *L. pholis* may enable larvae to synchronize settlement to optimal environmental conditions (Spounagle & Cowen, 1994).

In summary, this study found significant regional biological differences in pelagic larval duration and somatic growth rate of *L. pholis* showing a latitudinal pattern along a moderate

(1.8°C) sea surface temperature gradient. A consistent size at settlement was found in all sampled sites which emphasizes the fact that fish need to reach a minimum size to begin to settle. Longer planktonic periods in northern waters than in
southern waters suggest that slow-growing juveniles remain in the plankton until they reach appropriate size, perhaps in response to environmental conditions, namely due to sea water temperature exposure. However, given the small dataset and a few uncertainties of some methodological aspects of the present approach, these results should be interpreted with caution. More information about the movement
patterns, population structure and habitat connectivity in *L. pholis* is needed in order to improve the scientific knowledge about this species.

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3.2. Age, growth and sex of the shanny, *Lipophrys pholis* (Linnaeus, 1758) (Teleostei Blenniidae), from the NW coast of Portugal.

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Abstract

Age, growth, sex and gonadal maturation of the shanny, *Liphorys pholis* were determined in specimens caught on a rocky intertidal area in northern Portugal in order to provide essential ecological data about the species. This study represents the first available reference in the literature for *L. pholis* in a European southern location. Two hundred and fifty one individuals (115 females, 99 males and 37 undetermined; total length: 30-172 mm) were collected seasonally (November 2013, March 2014, June 2014 and September 2014) in a northern Portuguese rocky beach (Póvoa do Varzim: 41°23'47.79"N; 08°46'45.48"W) with a fine mesh aquarium fish net during the low tides. Marginal increment analysis was shown that one translucent and one opaque zones were formed each year in the sagittal otoliths. The *L. pholis* age ranged from 0 to 6 years. Males were larger and older than females. The parameters of the von Bertalanffy growth equation considering all individuals were $L\infty$ = 184 mm, K= 0.26 mmyear^{-1}, t0 = -1.34. The annual variation of the fish condition and hepatossomatic indexes appears to be related with the mobilization of the somatic reserves prior to reproduction. Maximum gonadosomatic index for males and females coincided with the breeding season (November and March). The sex-ratio was close to 1:1 with all maturity stages included. Furthermore, some stages of sexual development were observed during the same season in males and females, and several germinal cells were observed also at the same time within a

single ovary or testis, indicating that *L. pholis* is an asynchronous and multiple spawner. *L. pholis* appears to mature for both sexes around total length 70 mm and before reaching one year old.

Introduction

The shanny*, Lipophrys pholis* (Linnaeus, 1758), is a common intertidal fish found in the NE Atlantic, being recorded from Mauritania to Norway, including the Azores and Madeira Islands. It is also recorded in the Mediterranean Sea (Zander, 1986; Almada et al., 2001).

In the Portuguese coastal waters, *L. pholis* breeding season occurs from early autumn (early October) to middle spring (late May) and nests containing eggs can be easily observed in the rocky beaches (Faria et al., 1996). During the breeding period the males establish territories in crevices and stones where spawning takes place (Almada et al., 1990). The nests contain 3 to 8 batches of eggs from a single or multiple females deposited at different times during the course of a breeding season (Qasim, 1956, 1957). It is also known that *L. pholis* males are capable of multiple spawning episodes (Ferreira et al., 2011). According to captive experiments, embryonic development lasts 16 days at 17ºC (Faria et al., 2002). After hatching, the pelagic larvae disperse to the coastal area and, two to three months later, individuals return and settle in the intertidal pools, around early winter (Faria et al., 1996).

Recently, a field study using otolith microstructure of *L. pholis* early juveniles showed a pelagic larval duration ranging from 57 to 73 days post-hatching, however, with a general shortening along the Portuguese coast from North to South, mainly due to the regional water temperature differences (Carvalho et al., 2016). Furthermore, the same work revealed that fish need to reach a minimum size (TL ~ 19 mm) to begin the settlement process (Carvalho et al., 2016). After metamorphosis and settlement, characterized by pronounced morphological and physiological changes, early juveniles show a typical behavior associated with a benthic mode of life (Qasim, 1957; Faria and Almada 2001; Faria et al., 2002). Recruitment of fishes < 20 mm ceases three months after the end of the breeding season (Faria et al., 1996). In Portugal, the early juveniles can growth almost without interruption during the warmer months and those who are recruited in early winter are able to reach the minimum size, being sexually mature within one year (Faria et al., 1996).

L. pholis has been studied intensively in the northern parts of its geographical range, especially around the British Isles. Here the breeding season appears to be shorter and spawning takes place during spring and early summer (March/April to August) (Qasim, 1957). Furthermore, age and length relationships, determined from otolith readings of individuals collected in the British and Irish waters, showed that both sexes appear to grow at the same

rate, and that individuals do not reach in general more than six years of age (Qasim, 1957; Bowers, 1960; Dunne, 1977).

This study presents data on age, growth, sex-ratio and sexual developmental stages of *L. pholis* captured in the north coast of Portugal. Ultimately, the hereby study intents to test if these basic biological traits which are, at present, unknown for this species in the southern locations, also differ from the northern geographic locations.

Material and methods

Fish collection

Four sampling campaigns were conducted seasonally from November 2013 to September 2014 in a rocky beach located in Póvoa do Varzim, north of Portugal (41°23'47.79"N; 8°46'45.48"W) (Table 1). A total of 251 individuals were randomly collected during the low tides with hand nets, transported to the laboratory in isothermal containers and killed with a lethal dose of 2-phenoxyethanol.

Table 1. Sampling season, collection date, total length (TL: Mean ± SE) and age groups (Years) for *Lipophrys pholis* specimens used in this study and collected from November 2013 to September 2014 in a Portuguese northern rocky beach (Póvoa do Varzim).

Season	Collection Date	Sex	N	TL (mm)	Age Group
Autumn	05.11.2013	Females	15	51 ± 2	0
			4	71 ± 5	1
			3	116 ± 5	2
			4	120 ±5	3
			2	131 ± 6	4
			0	-	5
			2	139 ± 1	6
		Males	7	54 ± 3	0
			2	69 ± 6	1
			6	111 ± 4	2
			2	125 ± 0	3
			4	143 ± 6	4
			2	145 ± 5	5
			2	152 ± 21	6
		Undetermined	3	55 ± 0	0
Winter	18.03.2014	Females	19	65 ± 2	0
			7	84 ± 2	1
			6	107 ± 3	2
		Males	10	62 ± 3	0
			7	86 ± 2	1
			2	123 ± 7	2
			1	120	3
		Undetermined	13	44± 3	0
Spring	16.06.2014	Females	-	-	0
			21	78 ± 2	1
			3	102 ± 6	2
			4	124 ± 3	3
		Males	1	60 ± 1	0
			11	76 ± 2	1
			6	100 ± 3	2
			1	105	3
		Undetermined	15	52 ± 1	0
Summer	10.09.2014	Females	6	51 ± 2	0
			9	84 ± 3	1
			7	106 ± 3	2
			3	130 ± 4	3
			1	145	4
		Males	13	54 ± 2	0
			4	91 ± 1	1
			8	110 ± 3	2
			6	130 ± 4	3
			1	150	4
			2	154 ± 1	5
		Undetermined	6	46 ± 3	0

Total length (TL, to the nearest 1 mm), weight (W, to the nearest 0.1 g), gonadosomatic (GSI) and hepatosomatic (HSI) index were measured for all fish sampled. Sagittal otoliths were removed from the cranial cavity, cleaned from adherent tissues and stored dry in labelled Eppendorfs for ageing purposes. One small medial portion from each gonad was used for further histological analysis.

Fish condition indexes

The GSI and HSI indexes were calculated following the formulas GSI = $(W_G/W_E) \times 100$ and HSI = $(W_L/W_E) \times 100$, where WE is the eviscerated fish, and W_G and W_L are the gonads and liver weights, respectively.

A weight length relationship was stablished for the females (n=115) and for males (n=99). The Fulton´s condition factor (K) was calculated for each individual according to the equation: $K = 100(W/TL^3)$.

Sex determination and gonadal development stages

The sex was determined, if possible, through gonad visual identification and later confirmed by histological observations. The gonads from *L. pholis* individuals were fixed in Bouin liquid, dehydrated through graded alcohols, embedded in paraffin wax and sectioned at 5–7 µm. Sections were stained with haematoxylin–eosin, examined at 40-1000 X by light microscopy (Olympus, CX41). Microphotographs were taken with a digital USB camera (Olympus, SC 30) in order to assign sex and gonadal maturity stages based on previous works (Qasim, 1955; Ismail et al., 1990; Santos, 1995; Ferreira et al., 2011, Ferreira et al., 2012). Individuals in which it was impossible to identify the sex and developmental stage were considered undetermined.

Ageing fish and growth curves

To determine the age, the whole otoliths were immediately immersed in a clearing mixture after extraction (ethanol: glycerol; 1:1) and photographed with a stereomicroscope (Meiji, EMZ-13TRX) coupled to a 3.3 megapixel color CMOS camera (Olympus, SC30) at 200X and 400X magnifications. The quality of the digital images was improved using a free software (Paint.NET v3.5.10). After image software acquisition, growth annuli from right and left otoliths were blind count by two experienced readers. When the readers agreed on an age for both

otoliths, that age was assigned to the fish. Furthermore, the expected radius of the otolith of the young of the year (along the axis used for ageing) at the time of annulus formation was used to determine the expected radius of the first annulus (Carvalho et al., 2016). This approach requires an estimate of mean YOY fish length in the season of annulus formation (e.g. around the first birthday). When this estimate is inserted into a fish length-otolith length regression, the expected mean diameter of the first annulus can be predicted. It then becomes a simple matter to overlay the expected annulus diameter on probable first annuli in the otolith (Campana, 2001). The age was calculated by counting the translucent bands, but taking into consideration the date of birth (January 1st, following the rules in the northern hemisphere) and the date of capture (Panfili et al., 2002).

Marginal increment analysis was used to validate the annual growth pattern of periodicity deposition. The type of the band recorded in the edge of the otoliths (translucent or opaque), expressed as percentage and the measurement from the last annulus to the otolith edge, were used for the evaluation of the marginal zones over time (Panfili et al., 2002).

The von Bertalanffy growth curve (VBGC) parameters (Linf, k and t0) were estimated from the length-at-age data using a nonlinear least-squares function (Everhart et al., 1975). Likelihood ratio tests were used for comparison of the males and females VBGC (Kimura, 1980). VBGC parameters and comparison between curves were performed using the Package Fishmethods for R software.

Total length (L50) and age (A50) at first maturity were determined using the mature individuals (maturing + spawning + spent stages) collected during the species known breeding season (November 2013 and March 2014). The initial maturity status data were converted into a binomial dataset (immature 0, mature 1). TL was binned by 5 mm. A logistic regression was calculated by sex for L50 and A50 following the equation $Y = 1/[1 + e^{-(a+bx)}]$, where X is the TL (or Age) and Y is the proportion of mature individuals. L50 (or A50) was calculated as $-a/b$ (Mollet et al., 2000). An F-test known as the analysis of residual sums of squares (ARSS) was used to check for statistically significant differences between sexes (Ainsley et al., 2011).

Statistical analysis

Data were tested for normality and homogeneity of variances prior to statistical analysis. Significant differences were determined by One-Way Analysis of Variance (One-Way ANOVA) followed by a Tukey test, if needed. Total length within each age group was compared using a Student T-test. For the statistical procedures a level of significance of 0.05 was used. Results were presented as means ± standard errors. Statistical analysis was performed using Sigmaplot (version 11.0).

Results

The total length and weight of *L. pholis* ranged from 30 to 172 mm and 0.2 to 51 g, respectively. The results showed, in overall, that males were larger and heavier (TL= 92 ± 3 mm; W= 10 ± 1 g) than females (TL = 83 ± 2 mm; W= 7 ± 1 g) and undetermined fish (TL = 48 ± 1 mm; W= 1 ± 1 g) in our collection. However, no significant differences were detected in length and weight for fish within each age group (t-student, $P<0.05$).

The GSI, HSI and K values for females, juveniles and adults, are given in Fig. 1A and Fig 1B, respectively.

GSI varied significantly among seasons for female juveniles (One-Way ANOVA, $F_{3,58} = 8.856$, $P < 0.05$) namely between winter and the other seasons (Tukey Test, $P < 0.05$) (Fig. 1A). For female adults GSI also varied among seasons (One-Way ANOVA, $F_{3,50}= 3.761$, $P < 0.05$), namely between winter and spring (Tukey Test, $P < 0.05$) (Fig. 1B).

HSI varied significantly among seasons for female juveniles (One-Way ANOVA, $F_{3,58} = 8.242$ $P < 0.05$), namely between summer and spring and summer and autumn (Tukey Test, $P<0.05$) (Fig. 1A). For female adults HIS did not vary significantly among seasons (One-Way ANOVA, $F_{3,50} = 1.503$, $P < 0.05$).(Fig. 1B).

For female juveniles K were significant different among seasons (One-Way ANOVA, $F_{3,58} = 3.634$, $P < 0.05$), namely between spring and summer (Tukey Test, $P < 0.05$) (Fig. 1A). For female adults K were significant different among seasons (One-Way ANOVA, $F_{3,50} = 3.407$, $P < 0.05$), namely between winter and spring (Tukey Test, $P < 0.05$) (Fig. 1B).

The GSI, HSI and K values for males, juveniles and adults, are given in Fig. 1C and Fig 1D, respectively.

GSI did not varied significantly among seasons for male juveniles (One-Way ANOVA, $F_{3,43} = 0.613$, $P > 0.05$) (Fig. 1C). For male´s adults GSI also varied among seasons (One-Way ANOVA, $F_{3,48}= 6.891$, $P < 0.05$), namely between autumn and spring (Tukey Test, $P < 0.05$) (Fig. 1D).

HSI for juvenile males varied significantly among seasons (One-Way ANOVA, $F_{3,43} = 3.049$, $P < 0.05$), namely between summer and spring (Tukey Test, $P<0.05$) (Fig. 1C). For male adults HSI also varied significantly among seasons (One-Way ANOVA, $F_{3,48} = 4.614$, $P < 0.05$), being autumn significantly different from winter and spring (Tukey Test, $P<0.05$) (Fig. 1D).

K were not significant different among seasons for male juveniles (One-Way ANOVA, $F_{3,43} = 0.554$, $P < 0.05$) (Fig. 1C). For the male adults K was significant different among seasons

(One-Way ANOVA, $F_{3,48}$ = 7.634, P< 0.05), namely between autumn and the other seasons (Tukey Test, P<0.05) (Fig. 1D).

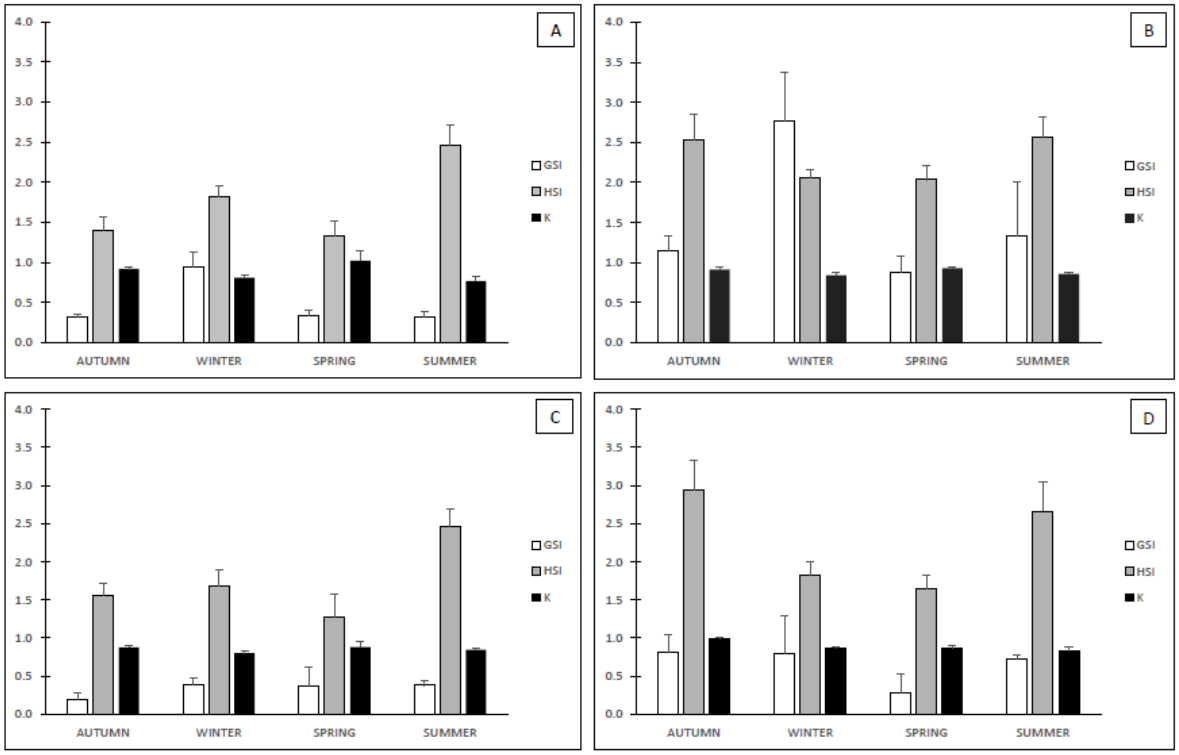

Fig 1. Seasonal variation of the gonadossomatic index (GSI), hepatossomatic index (HSI) and Fulton's condition factor (K) for the juvenile (A) and adults females (B), and for the juveniles (C) and adults (D) males of *Lipophrys pholis* used in the present study. Juveniles represents the immature and developing individuals. The adults represent the maturing, spawning and post-spawning stages. Y and X axes presents respectively the numeric values and the seasonal collection date. The values represent the mean ± SE.

Macroscopically, different gonadal developmental stages were observed in females, with ovaries varying from transparent to bright orange and with spherical golden-brown oocytes when ready to spawn. When spawning occurs, the ovaries become shrunken and colored cream. The ovaries contained different types of germ cells: oogonias, perinuclear oocytes; cortical alveolar oocytes, early vitellogenic oocytes and vitellogenic oocytes. The analysis of histological sections showed that females contained five stages of ovarian development: I - immature, II - maturing or early oogenesis; III - mature or mid-oogenesis, IV - ripe or spawning and V - spent. Immatures females were recorded during all year, early oogenesis was recorded mostly during September, mid-oogenesis mainly during November, spawning occurring in November and March and spent during June (Fig. 2A).

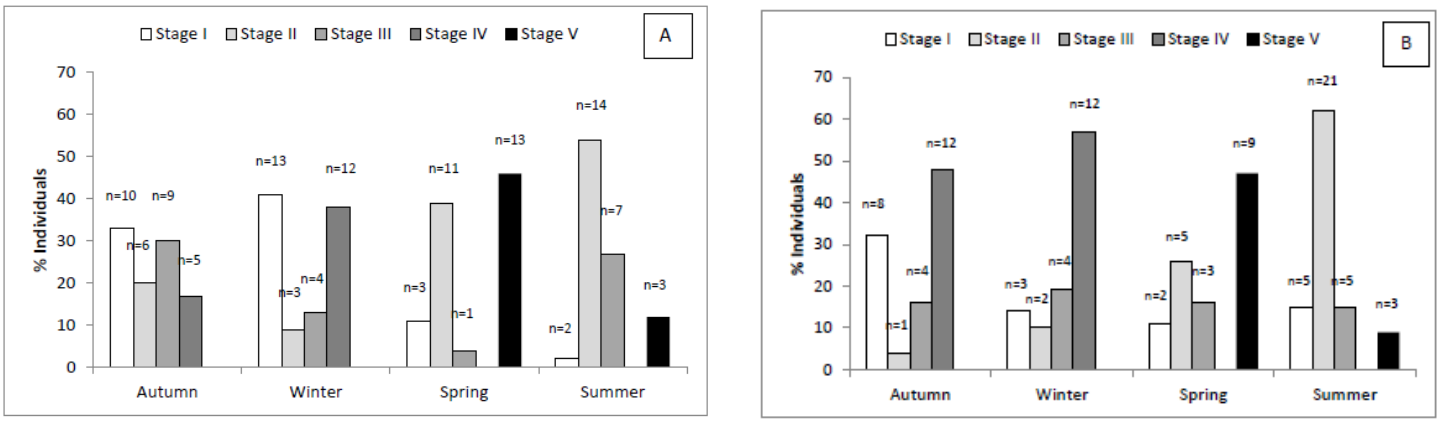

Fig 2. Seasonal variation (in percentage and absolute values in y axis and above columns, respectively of the sexual developmental stages through the sampling seasons for (A) females and (B) males of *Lipophrys pholis* used in the present study. Stages I: Immature, II: Maturing; III-Mature, IV: Spawning and V: Spent.

The immature stage was characterized by the exclusively existence of primary germ cells in the ovary (Fig. 3A). The early oogenesis stage was characterized for oogonia that appeared isolated or in small groups, being the perinuclear oocytes especially abundant during this stage. These cells were characterized by a highly basophilic cytoplasm with the presence of a nucleus containing several nucleoli and a thin follicular layer surrounding the oocytes (Fig. 3B). Mid-oogenesis was mainly characterized by the presence of cortical alveolar oocytes with appearance of a typical vacuolization pattern (cortical alveoli) in the cytoplasm, with the zona radiata and the theca perfectly visible. The early vitellogenic oocytes were also found in this stage and were characterized by an increased centrifugal accumulation of vitellogenic yolk granules that tend to dislodge the cortical alveolar material to the periphery of the cytoplasm (Fig. 3C). In the spawning stage, ovaries were mainly occupied by vitellogenic oocytes but also oocytes in all development stages (Fig. 3D). The spent stage was characterized by the existence of residual oocytes and perinuclear oocytes in the ovary (Fig. 3E), indicating the upcoming onset of the breeding season.

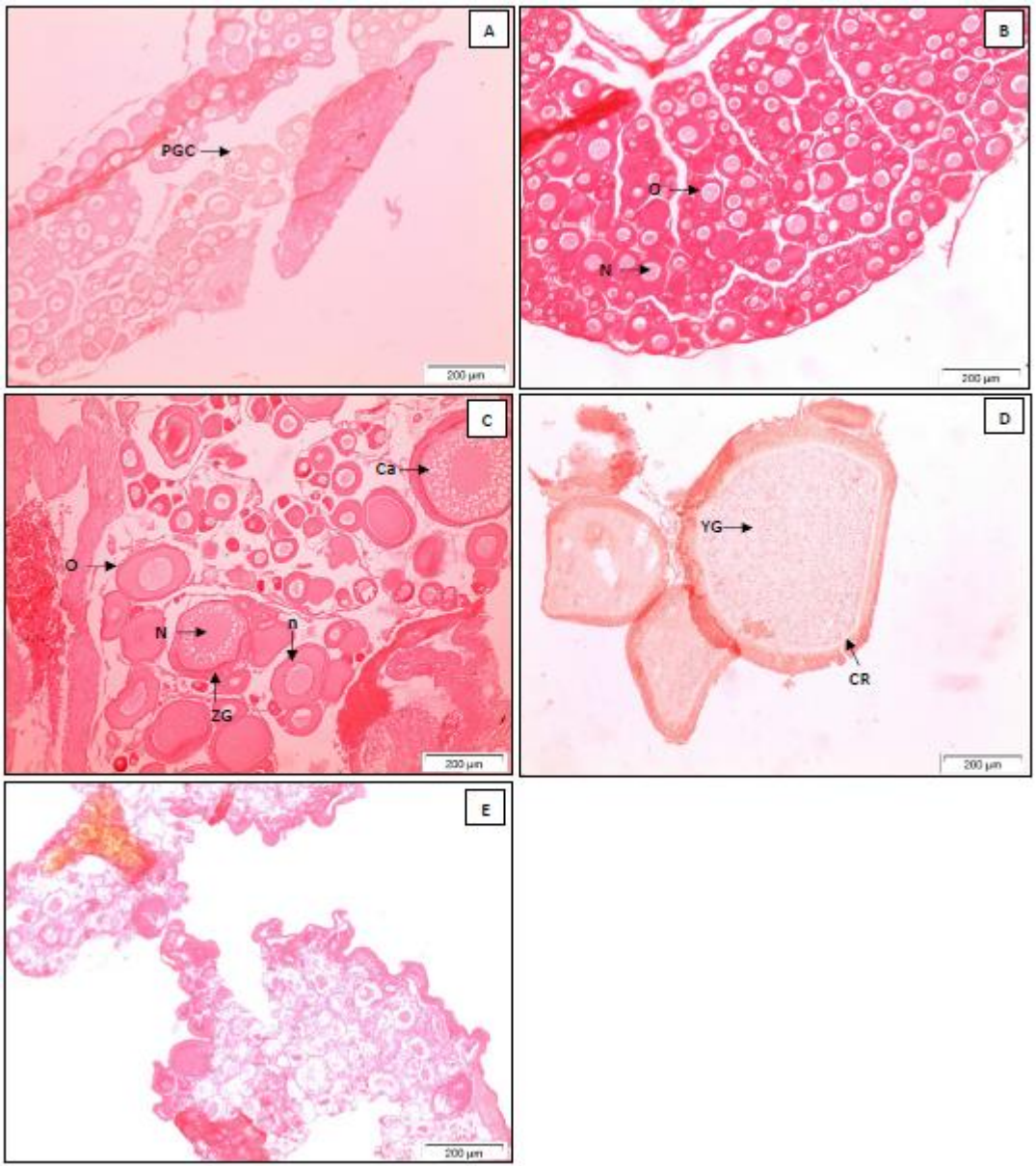

Fig.3 Histological cross sections through the ovaries of five females of *Lipophrys pholis* used in this study showing the five developmental sexual stages: A. immature stage (TL=57 mm and GSI= 0.05); B. early oogenesis stage (TL=64 mm and GSI=0.34); C. mid oogenesis stage (TL=125 mm and GSI=1.10); D. spawning stage (TL=120 mm and GSI=1.10); and E. spent stage (TL=90 mm and GSI=0.40). Primary germ cells (PGC), oocyte (O), nucleus (N), nucleoli (n), cortical alveoli (Ca), corona radiata (CR), fully hydrated oocytes with yolk platelets (YG) and zona granulosa (ZG). Hematoxylin-Eosin. Magnification 100 X.

Through visual identification of the male gonads, testes are paired elongated bodies, consisting of tubules of the unrestricted spermatogonial type. When immature, their color is transparent, changing to milky white as maturation occurs. After spawning the gonads become much shrunken and greyish. Male gonads are composed by two main components: the testis and the testicular gland. It has connection to the tubules and the vas deferens. The testes contain different cell types: spermatogonia, primary spermatocytes, secondary spermatocytes,

spermatids and spermatozoa. According to the frequency of testicular components, five stages of gonadal maturation were also found in males: I - immature, II - maturing or early spermatogenesis, III - mature or mid spermatogenesis, IV- ripe or spawning and V - spent. Immature individuals were recorded during all year, early oogenesis was recorded mostly during September, mid oogenesis during November, spawning occurring in November and March and spent during June (Fig. 2B). Similarly to females, the immatures males were recorded during all year. This is characterized by the exclusively existence of spermatogonias in the testis (Fig. 4A). The early spermatogenesis was characterized mainly by the presence of spermatogonia cells (Fig. 4B) and by the proportionally bigger size of testicular gland compared to testis. Mid spermatogenesis was characterized by the reduction of spermatogonia cells while the proportion of primary and secondary spermatocytes increased (Fig. 4C). In this stage testes increased in size. In the spawning stage, the spermatids increased and the seminiferous tubules were full of spermatozoa, which became the predominant cell type (Fig. 4D). In the spent stage spermatozoa remained in the testis (Fig. 4E) and in testicular gland. In this stage, the testicular gland was proportionally wider than the testis with the existence of spermatogonia cells, indicating the beginning of the following breeding season.

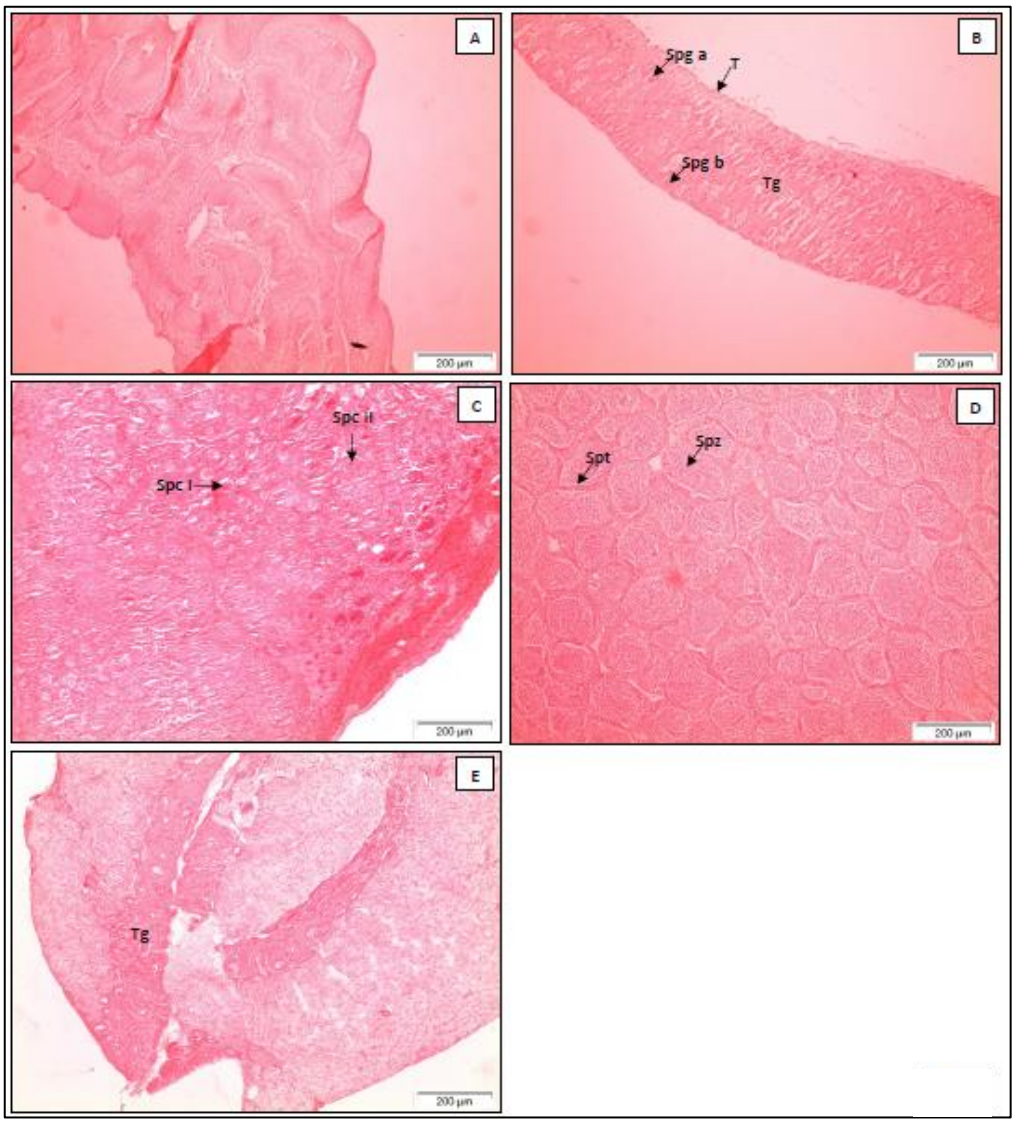

Fig. 4 Histological cross sections through the testis of five males of *Lipohrys pholis* used in this study showing the five developmental sexual stages: A. immature (TL=74 mm and GSI=0.12), B. early spermatogenesis (TL=66 mm and GSI=0.12), C. mid spermatogenesis (TL=75 mm and GSI= 0.90), D. spawning (TL=120 mm and GSI=1.10), and E. Spent (TL=100 mm and GSI=0.18). Spermatogonia type A (Spg a), spermatogonia type B (Spg b), spermatocyte I (Spc I), spermatocyte II (Spc II), spermatids (Spt), spermatozoa (Spz), testicular gland (Tg) and testis (T). Hematoxylin-Eosin. Magnification 100 X.

Sagittal otoliths observed under visible light showed clear opaque and translucent bands (Fig. 5).

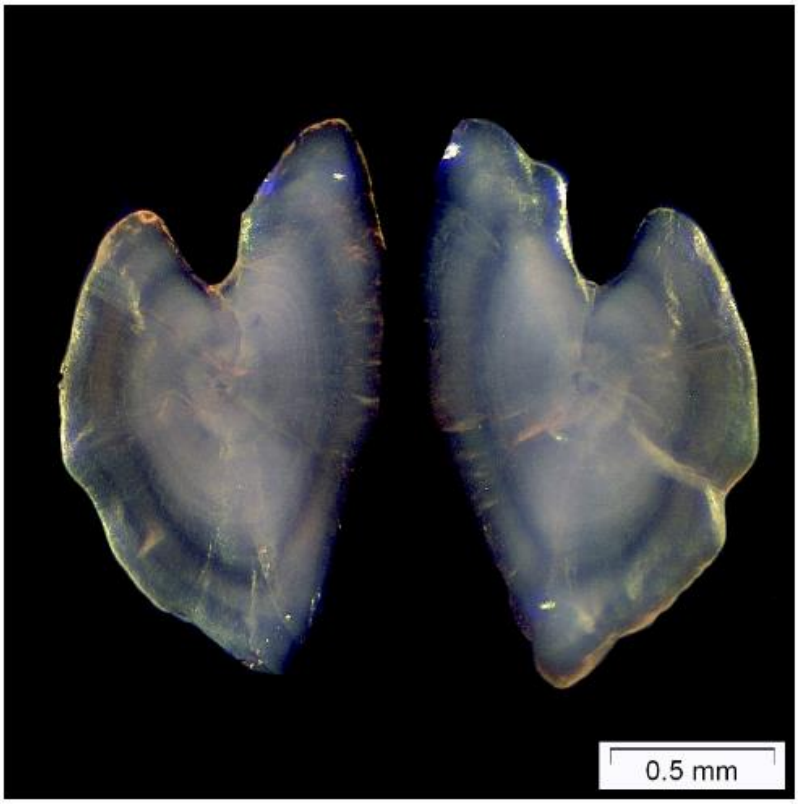

Fig 5 *Lipophrys pholis* otoliths (left and right, respectively) from an individual (TL = 105 mm) belonging to the age group 2 collected in the autumn. The winter zones can be seen as dark bands.

The nature of the otolith edge, opaque or translucent, and the marginal absolute distance, showed a seasonal frequency shift for the warmer seasons. Furthermore it appears that a single opaque zone is deposited each year during the warm season (spring/summer) (Fig. 6).

In this study the overall age for *L. pholis* ranged from 0 to 6 years (Table 1). Age group 0 was the most abundant (43%: TL =54±1 mm), followed by age group 1 (26%: TL = 79 ± 1 mm). The age group 2 recorded 16% (TL = 109 ± 0 mm), while age group 3 registered 8% of the total sample (TL = 123 ± 1 mm). The oldest age groups were poorly represented. 3% of individuals belonged to age group 4 (TL = 143 ± 2 mm) and 2% to age group 5 (TL =149 ± 1 mm)]. Only four individuals (two males and two females) belonged to age group 6 (2%; mean L_T= 145± 9 mm). The results also show that the hereby study happened to catch older males (males: 2.42 ± 0.14 years; females: 2.14 ± 0.11 years) during the fish collection.

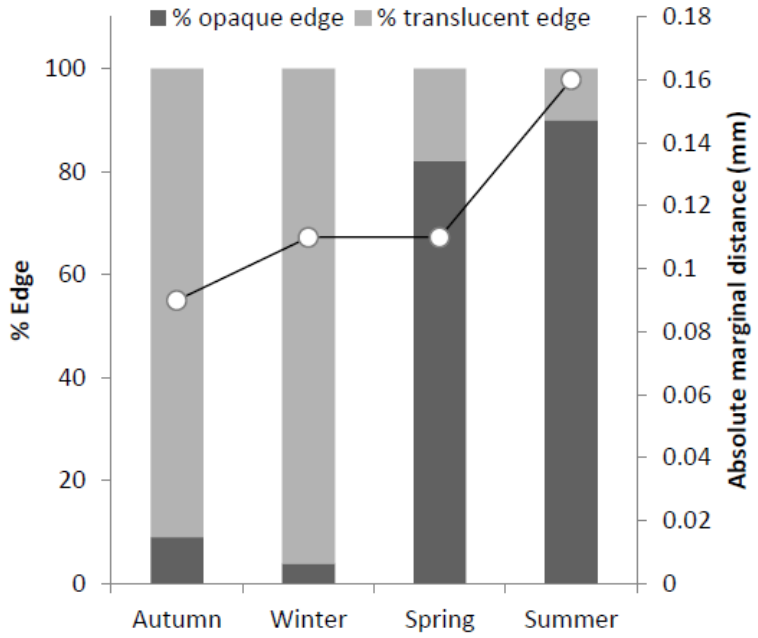

Fig 6 Evolution over the time of the percentage of opaque-translucent edges (column bars) and absolute marginal distances (open circles) in otoliths of *Lipophrys pholis* collected from November 2013 to September 2014 in a Portuguese northern rocky beach (Póvoa do Varzim).

The parameters of the VBGC considering all individuals, including the undetermined individuals, females and males could be found in Fig. 7A, Fig. 7B, and Fig 7C, respectively. However, there are no statistical differences (Linf, k and t0) between females and males ($P > 0.05$).

The maturation ogive (i.e. logistic regression curve fitted to estimate length at 50% maturity) showed that females reached L50 at 71 mm (Fig. 8A: $r^2 = 0.98$, $P < 0.05$, $n = 15$) and males at 68 mm (Fig. 8B: $r^2 = 0.99$, $P < 0.05$, $n = 20$). The results of the F-test showed significant difference in L50 between sexes ($F_{14,19} = 8.48$, $P < 0.05$). Females reached A50 at 0.7 years (Fig. 8C: $r^2 = 0.99$, $P < 0.05$, $n = 6$) and males at 0.4 years (Fig. 8D: $r^2 = 0.99$, $P < 0.05$, $n = 7$). Moreover, there was no significant difference in A50 between sexes ($F_{6,5} = 2.94$, $P = 0.12$).

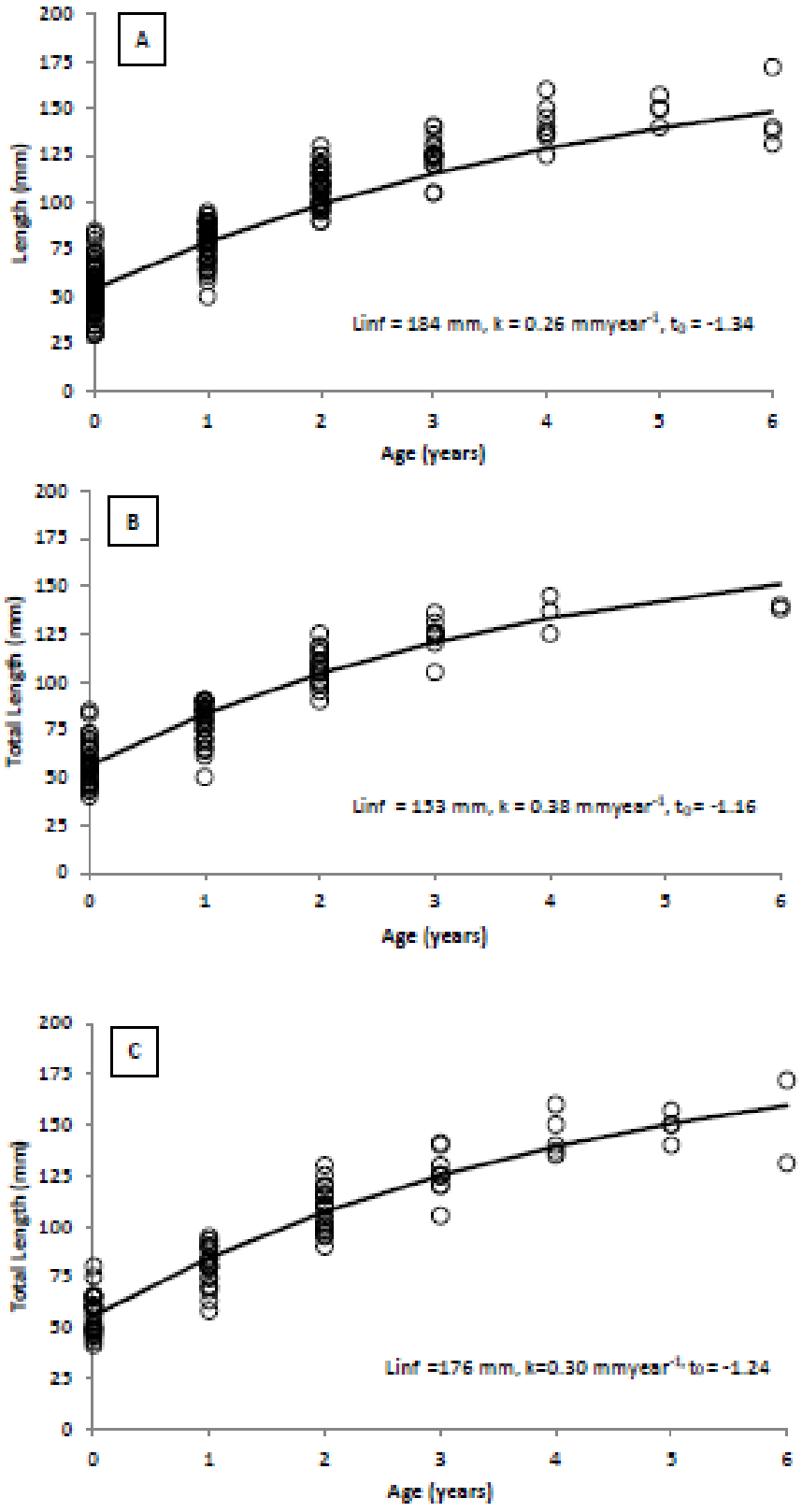

Fig 7 Graph showing the von Bertalanffy curves (A: total individuals, n=251; B: females, *n*=115; and C: males, n =99) and parameters (Linf, K and T_0) fitted for the *Lipophrys pholis* individuals collected in a northern rocky beach (Póvoa do Varzim) from November 2013 to September 2014.

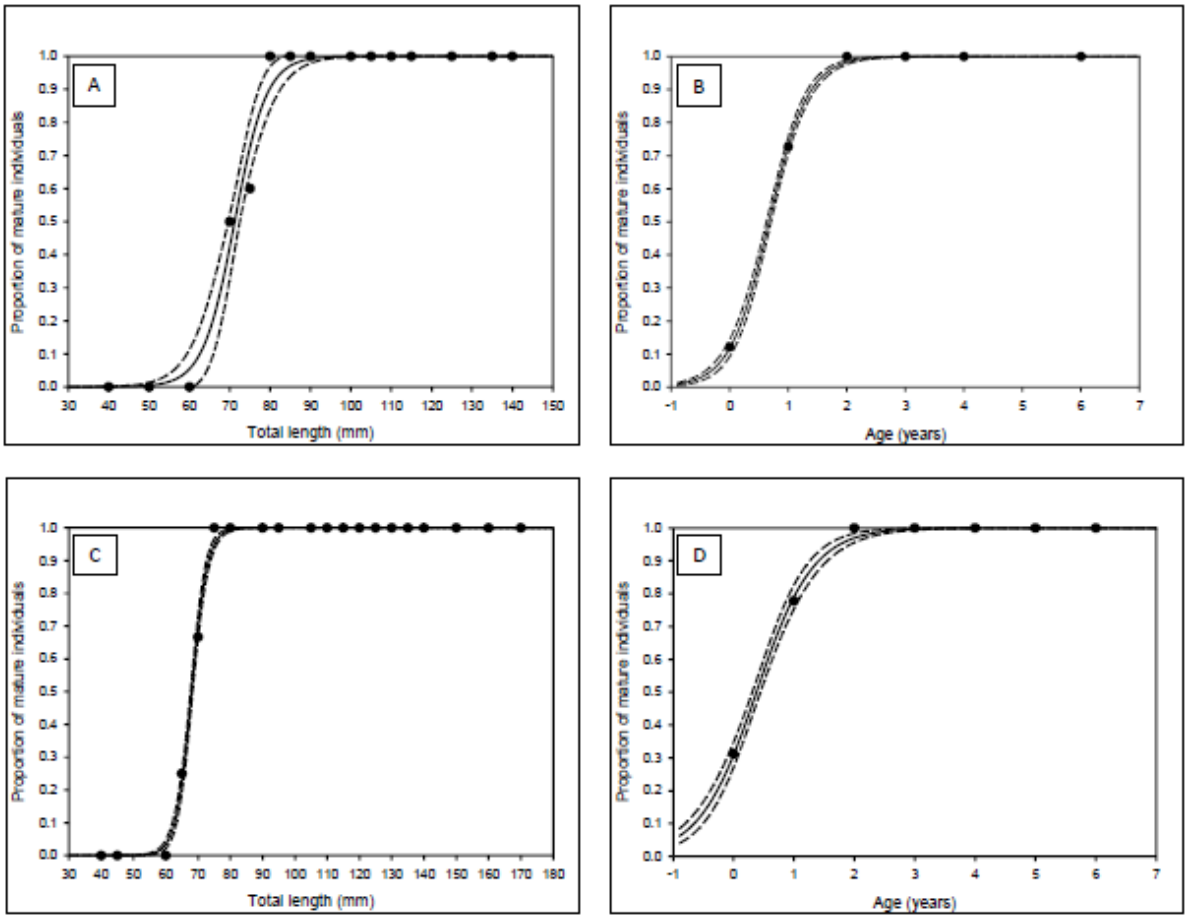

Fig. 8. Estimated total length (L50) and age (A50) at first maturity for female: A and B respectively and estimated total length(L50) and age (A50) at first maturity for male: C and D, respectively for of *Lipophrys pholis* used in this study with 95% confidence intervals depicted by dashed lines.

Discussion

GSI seemed to provide a good indication of the reproductive status of females and males of *L. pholis.* The maximum and minimum GSI values for both adult females and males were recorded simultaneously during November and March, which also coincided with the higher percentage of spawning individuals. Observations from previous studies (Ferreira et al., 2011 and 2012) corroborate these findings, since most females and males are ready to spawn in November. Lower observed GSI values were also recorded in June, as in the hereby study.

Liver plays an important role in storage energy before and in the onset of the reproduction season in fish (Zahnd, 1959). The mobilization of the somatic energy reserves needed for the reproductive development appear to begin for juveniles during the summer season showing a higher HSI. When lipid reserves are fully stored (autumn season in adults of both sexes), the needed for food intake reduces gradually through the year until the spring season, which

records the lowest HSI values. In *L. pholis* females, as in fish in general, it is well known that lipids deposited in the oocytes during maturation do not come directly from ingested food, but are transferred from the liver (Shackley and King 1977, 1978, 1979). For males, the lower HSI values recorded on late breeding season (March) could be supported by previous parental care studies which showed that for *L. pholis* there is a reduction in the feeding activity during the breeding season, since they spend more time guarding the eggs instead of feeding (Qasim, 1956; Almada et al., 1992; Gonçalves, 1997).

K was higher in spring and autumn for mature females and males, respectively, but lower in summer, for both sexes. K did not vary through the year for juvenile males, although, was also higher in spring for immature females. The seasonal variation was, however, inconclusive. It is well-known that K could reflect the state of sexual maturity and degree of nourishment, while may also vary with age, and in some species, with sex (Williams, 2000).

Marginal increment analysis has been successfully used in the present study as a semi-direct validation method for the annuli deposition in *L. pholis* otoliths. It appears that an opaque zone is laid down each year during the spring/summer season. It is a widespread technique to determine the timing of annual increment formation in adult fish due to its moderate sampling request and low cost (Campana, 2001). Age for *L. pholis* ranged from 0 to 6 years. The hereby estimates of ages and size frequencies were compared with a previous work occurring in west coast of Portugal (Faria et al., 1996). The fish sizes in the previous work were lower for age group 0 (42.1 mm), but higher for age group 1 (87.3 mm). For the subsequent age groups the reported sizes were similar (2: 106.1 mm; and 3: 125.3 mm). For older age groups, the fish sizes were only possibly to compare with a study made in English waters (Qasim, 1957). This study reported, in general, lower sizes for each age group (4: 133.0 mm; 5: 141.3 mm), with exception of the aged individuals (6: 153.0 mm).

The VBGC estimates from the otoliths in our study gave the result of Linf = 153 mm, k = 0.38 mmyear^{-1} and t0 = -1.16 and Linf = 176 mm, k= 0.30 mmyear^{-1} and t0= - 1.24 for females and males, respectively. However, no statistical differences were observed for the VBGC between sexes. Unfortunately there is no other published data for this species, but information for other small blenny fish concerning the VBGC estimated growth parameters can be found: Linf = 102 mm and t0=-1.62, or Linf = 97 mm and t0 = -0.77 for females and males of *Omobranchus punctatu* respectively (Ismail & Clayton, 1990); and Linf = 132 mm; k = 0.62 mmyear^{-1}; t0=-0.39 for *Parablennius ruber* (Azevedo and Homem, 2002).

Histological observations showed that almost all stages of gonadal sexual development were presented simultaneously in the ovaries and testes through the year. The observed five main stages of maturity corresponded to the existent literature for this species (Ismail et al., 1990; Qasim, 1957, Ferreira et al., 2011; 2012).

For females, the highest proportion of vitelogenic oocytes was recorded in November and March. June was defined as spent stage, where vitelogenic oocytes and residual perinuclear oocytes were still found. Early oogenesis, characterized by the existence of perinuclear oocytes, was recorded in September. Middle oogenesis with ovaries showing cortical - alveolar and vitelogenic oocytes was observed in November.

In males, the highest proportion of spermatozoa was found in November and March, but in November middle spermatogenesis was also observed. Early spermatogenesis was mainly observed in September showing spermatogonias that later would mature to spermatids and spermatozoa. June was defined as the spent stage with both spermatozoa and spermatogonia cells in testis.

The hereby results suggest that *L. pholis* has an asynchronous oocyte development, which confirms that females are multiple spawners, with eggs being recruited in several batches during the breeding season. Similar results were observed for males that are capable of multiple spawning episodes. Such results also agree with previous studies on the species (Ferreira et al., 2011; 2012). Furthermore, the sex-ratio of L*. pholis* observed here was most likely 1 to 1 throughout the year. An overall 1:1 phenotypic sex ratio tends to appear to an evolutionary tendency of vertebrates (Karlin and Lassard, 1983)

In this study, individuals from both sexes with less than one year, with total length around 70 mm were already mature. The hereby data is according with other authors that reported that both sexes of *L. pholis* could be mature around one year old with 80 mm length in the Portuguese coast (Faria et al., 1996; Monteiro et al., 2005). In northerly populations with colder water maturation is achieved after a period of two years (Milton, 1983). Furthermore it seems that early maturity observed in *L. pholis* appears to be common in blennies. *Salaria pavo* also starts reproducing at a small size (46.2 ± 4.8 mm) (Vinyoles and Sostoa, 2007); *Parablenius ruber* individuals become sexually mature around 60 mm, a size that can be reached in less than one year (Azevedo and Homem, 2002); and the close related species *Parablennius gattorugine* becomes sexually mature at one year old (Dunne and Byrne, 1979). A similar situation has also been described for a small littoral goby fish of the Azores: *Gobius paganellus* is reproductively mature in their first year of life (Azevedo and Simas, 2000). However, since the age 0 individuals could be either mis-aged and/or biased to greater size lengths, these results should be interpreted with caution.

The results found in the present work showed that variation in some life traits of *L. pholis* may occur within its geographical scale along the western European shores.

Acknowledgments

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CHAPTER 4

Otolith microchemistry to study *L. pholis* movements

Movement, connectivity and population structure of the intertidal fish *Lipophrys pholis* (Linnaeus, 1758) as revealed by otolith oxygen and carbon stable isotopes

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Abstract

The shanny *Lipophrys pholis* is an intertidal fish commonly found in the Portuguese coastal waters. Spawning takes place from early autumn to mid spring, after which demersal eggs hatch and larvae disperse along the coast. Two to three months later, young juveniles return to the tide pools to settle. However information on fish movement, habitats connectivity and population structure is scarce for this species. 120 early juveniles (16 to 35 mm) were collected in April 2014 from six rocky beaches along the western and south Portuguese coasts (Agudela, Cabo do Mundo, Boa Nova, Peniche, Sines and Olhos de Água). $\delta^{18}O$ and $\delta^{13}C$ were determined by isotope-ratio mass spectrometry. Data were analysed to determine whether isotopic signatures could be used to assess the degree of separation between individuals collected from different locations. Mean $\delta^{13}C$ and $\delta^{18}O$ values ranged from -0.02‰ to 1.14‰ and - 7.77‰ to -6.62‰, respectively. Both seawater temperature and salinity caused differences in otolith $\delta^{18}O$ among the four main sampling regions. The inter-regional variation in $\delta^{13}C$ was most likely related to slight differences in the diet, growth and/or metabolism of

fish. The distinct isotopic signatures, at least for the northern and central regions, suggested low levels of connectivity across large spatial scales during the juvenile stage. Furthermore, similar isotopic signatures within the same region indicated some degree of larval oceanic retention at short-spatial scales. This study suggests that stable isotope records in otoliths could provide new insights about home residency, movements and habitat connectivity of intertidal fish populations.

Key-words: Blenniidae, Sagittae, Isotopic Signatures, Fish Life History.

Introduction

The shanny *Lipophrys pholis* is a resident fish of the intertidal rocky shores (Gibson 1982) and it is the most abundant blenniid along the Portuguese coast (Almada et al. 1990). This species is found in the NE Atlantic, from Mauritania to Norway, including the Azores and Madeira Islands, and also in the Mediterranean Sea (Zander 1986, Almada et al. 2001). In Portuguese coastal waters, *L. pholis* spawning season occurs from early autumn to mid spring (October/November to May) (Faria et al. 1996), and embryonic development stage lasts 16 days at 17°C (Faria et al. 2002). After hatching, pelagic larvae disperse along the coastal area and early juveniles (<20 mm) seem to return to a particular set of rock tide pools two to three months later, in early winter, to settle (Faria et al. 1996). Recently, it has been shown that pelagic larval duration along the Portuguese coast ranged from 57 to 73 days post-hatching showing a latitudinal reduction trend from North to South, probably due to regional differences in water temperature (Carvalho et al. 2017). Furthermore, the same study revealed that fish need to reach a minimum size (~19 mm) to begin the settlement process. After metamorphosis and settlement early juveniles show a typical behavior associated with a benthic mode of life (Qasim 1957; Faria & Almada 2001b; Faria et al. 2002). In Portugal, early juveniles may grow almost uninterruptedly during the warmer months and fish recruiting in early winter are able to reach the minimum sexual maturity size within one year (Faria et al. 1996). A recent study showed that in the NW coast of Portugal the length and age at first maturity for both sexes occur around 70 mm and before reaching one year old, respectively (Carvalho et al. 2017). A mitochondrial DNA study of *L. pholis* found no significant population genetic structure along the Portuguese coast, attributing the genetic homogeneity of this species to an efficient gene flow as a result of the oceanic dispersal of the planktonic larvae (Francisco et al. 2006).

It is well-known that otoliths begin to form before hatching and grow continuously through the life cycle preserving a life-long record of fish environment (Campana 1999). Combined stable oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) otolith signatures have been successfully used as natural tags for fish population structure studies (Gao et al. 2004; Correia et al. 2011; Daros et al. 2016). $\delta^{18}O$ in otoliths are usually used as a proxy of ambient sea temperature (Thorrold et al. 1997; Radtke et al. 1996; Høie et al. 2004). $\delta^{13}C$ appear to be influenced by various factors, including fish metabolism and diet (Schwarcz et al. 1998; Høie et al. 2003; Gao et al. 2004). $\delta^{13}C$ may be also influenced by the isotope ratios of the dissolved inorganic carbon in the water (Thorrold et al. 1997; Solomon et al. 2006; Yoshimura et al. 2015).

In the present study $\delta^{18}O$ and $\delta^{13}C$ were analysed in whole otoliths of *L. pholis* early juveniles captured at six beaches along the western and south Portuguese coasts. The purpose of this work was to determine whether isotopic signatures of otoliths could be used to assess the degree of separation between regions, to investigate habitat connectivity between tidal pools and coastal waters and eventually to trace fish origin.

Material and methods

Fish sampling

Fish sampling took place in in April 2014 in four main regions about 300 km apart from each other along the northern and southern Portuguese coasts: North-West (Cabo do Mundo), Central-West (Peniche), South-West (Sines) and South (Olhos de Água). In the NW region we have also collected fish in two additional sites (Agudela and Boa Nova) about 3 km from each other (Fig. 1 and Table 1).

Individuals were captured with hand-nets during the low-tides in the rocky beaches, transported to the laboratory in isothermal containers and killed with a lethal dose of 2-phenoxyethanol. In the laboratory, fish total length (TL, 0.1 mm) and mass (M, 0.0001 g) were measured (Table I). A total of 120 early juveniles (20 per location), ranging from 16 to 35 mm of total length (TL: 20.0 ± 0.3 mm) were used for further analysis.

Sagittal otoliths were removed with plastic forceps and cleaned in an ultrasonic bath for 5 min in ultrapure water (Milli-Q-Water) followed by immersion in 3% analytical grade hydrogen peroxide (H_2O_2, Fluka TraceSelect) for 15 min to remove any adherent biological tissues. Finally otoliths were triple-rinsed with Milli-Q-Water to remove possible contaminations, air dried in a laminar flow cabinet, weighed (0.000001 g) and stored in dry Eppendorf tubes for isotopic analysis.

Fig. 1: Location of Portugal in the NE Atlantic ocean showing the *Lipophrys pholis* sampling regions (main map: black dots). Location of the northern sampling sites in the city of Matosinhos (inset map: black stars).

Otolith isotopic analysis

Otoliths were crushed into a fine powder using a stainless steel spatula. The crushed powder (20–40 µg) was analysed for stable oxygen and carbon isotopic composition using a Thermo Finnigan MAT253 mass spectrometer coupled to a Kiel IV carbonate preparation device. Three standards were used: the external standard NBS19 and the in house standard NFHS1 for both $\delta^{18}O$ and $\delta^{13}C$, and the external standard NBS18 for ^{13}C only. The reproducibility of all standards amounted to 0.1‰ and 0.05‰ (1 SD) for $\delta^{18}O$ and $\delta^{13}C$, respectively.

Water analysis

Water samples for analysis of oxygen isotopic composition (VSMOW) were also collected in April 2014, at each location, during the fish sampling, with a bucket on the coastal line, just below the surface, transferred to 12 mL glass vials and stored in the refrigerator. Water $\delta^{18}O$ values were determined by headspace analysis using a Thermo Finnigan Delta+ mass

spectrometer equipped with a GasBench-II preparation device. The long-term standard deviation of routinely analysed in-house water standards was <0.1‰ (1 SD).

Seawater surface temperature

Sea surface temperature (SST) data of the four main regions were obtained from floating Datawell coastal buoys of the Instituto Hidrografico da Marinha Portuguesa, located as close as possible to the sampling areas and anchored near the 100 m bathymetry (Leixoes CSA92/D : WGS 84 – 41°19'N, 8°59'W, depth: 83 m; Nazare CSA88/1D : WGS 84- 39° 33´N, 9° 12´W, depth: 88 m ; Sines CSA83/1D : WGS 84 - 37°55' N, 8°55.73'W, depth: 97 m; and Faro CSA82/D :WGS 84- 36°54' N, 7°53' W, depth: 93 m). SSTs were calculated for each individual weighted to the estimated age of fish to represents its lifetime. Fish age was obtained using the already published linear relationships between length and post-hatching days for the early juveniles of *L. pholis* in the same or nearby sampling regions (Carvalho et al. 2017). In the present study, the relationship between $\delta^{18}O$ values of otolith carbonate and SSTs was explored with the expectation that if separate fish groups exist the isotopic signature of their carbonate otoliths would be correlated with the water temperature in which they grow due to the temperature dependent fractionation of $\delta^{18}O$ during otolith calcification (Correia et al. 2011).

Sea surface salinity (SSS)

Historical seawater surface salinity data of the sampling areas were provided from the EEMA Project (Projeto de Avaliação do Estado Ecológico das Massas de Água Costeiras e de Transição Adjacentes e do Potencial Ecológico de Massas de Água Fortemente Modificadas) and APA (Agência Portuguesa do Ambiente). Data were collected between 16 and 23 March 2011 in the Portuguese coastal line, namely close to the four main regions of the present study. A probe was used to measure the salinity values through direct readings at 0.5 m just below the surface. SSS data have been shown a strong annual seasonality but highly consistent over the years (January 1958 – December 2001) along the Atlantic coast of the Iberian Peninsula (Lima et al. 2006)

Data analysis

After testing for normality (Shapiro–Wilk test, $P > 0.05$) and homogeneity of variances (Levene´s test $P > 0.05$), $\delta^{13}C$ and $\delta^{18}O$ values were analyzed by analysis of covariance (ANCOVA). Otolith mass is considered to be a proxy for fish age and growth rate and therefore it was used as a covariate in ANCOVA. Location was treated as a fixed factor. Since in the hereby data $\delta^{13}C$ was significantly related with otolith mass and to ensure that this correlation did not confound any site-specific differences in otolith chemistry, $\delta^{13}C$ was corrected by subtraction of the product of the common within-group linear slope multiplied by the otolith mass from the observed isotopic ratios (Gerard & Muhling 2010). However this procedure did not have any effect on the results, and did not change the significance of the initial data nor the corresponding discriminant analysis. A post hoc Tukey HSD test was used to examine the existence of any significant differences in the individual isotopic ratios of carbon and oxygen among the four sampling regions and between the three sampling sites. Multivariate analysis of variance (MANOVA) and quadratic discriminant function analysis (QDFA) were used to explore the variation of multi-isotopic signatures among regions (Cabo do Mundo, Peniche, Sines and Olhos de Água) and among sites (Agudela, Cabo do Mundo and Boa Nova). For MANOVA, we report the approximate F-ratio statistic for the most robust test of multivariate statistics (Pillai's trace). Pairwise comparisons after MANOVA were done using the Hotelling's T-square test. To allow a visual inspection of the individual isotopic signatures per group a bi-plot ($\delta^{18}O$ vs $\delta^{13}C$) was displayed. QDFA was used to re-classify individuals to sampling locations. Classification accuracies of the discriminant functions for each region and site were evaluated using the percentage of correctly classified individuals from jackknife testing (leave one-out cross-validation). Statistical analyses were performed using the software SYSTAT 12. Results are presented as means ± standard errors (SE). A significance level (α) of 0.05 was used for all statistical procedures.

Results

The mean otolith isotopic ratios obtained from the six sampling locations ranged from -7.77‰ to -6.62‰ for $\delta^{13}C$ and -0.02‰ to 1.14‰ for $\delta^{18}O$ (Table 1).

Table 1: Location, sampling date, number of specimens (N), fish total length (TL), otolith weight, carbon and oxygen otolith isotopic values, oxygen water isotope, sea surface temperatures (SST) and sea surface salinity (SSS). Values are expressed as means ± standard errors.

Geographic Location		Date	N	TL (mm)	Otolith weight (µg)	Otolith $\delta^{13}C$ (‰VPDB)	Otolith $\delta^{18}O$ (‰VPDB)	Water $\delta^{18}O$ (VSMOW)	SST (°C)	SSS (psu)
Agudela	41°14'23.80"N 8°43'33.19"W	28 April 2014	20	19.1±0.4	18.8±1.2	- 7.59±0.13	0.02±0.08	0.42±0.01	13.73±0.05	32.0±1.2
Cabo do Mundo	41°13'24.28"N 8°42'58.57"W	29 April 2014	20	21.4±0.9	22.4±1.7	- 7.38±0.15	0.06±0.06	0.39±0.01	13.73±0.05	32.0±1.2
Boa Nova	41°12'27.99"N 8°43'03.40"W	29 April 2014	20	19.0±0.8	20.9±4.0	- 7.77±0.14	0.01±0.04	0.38±0.01	13.73±0.05	32.0±1.2
Peniche	39°22'36.21"N 9°20'23.31"W	18 April 2014	20	19.6±0.2	18.6±0.4	- 6.62±0.14	1.14±0.12	0.83±0.07	13.90±0.05	35.5±0.2
Sines	37°53'7.22"N 8°47'43.16"W	5 April 2014	20	22.3±1.3	33.1±5.9	- 7.02±0.22	0.82±0.05	0.86±0.18	14.83±0.03	35.0±0.0
Olhos de água	37°05'26.44"N 8°11'00.33"W	14 April 2014	20	21.2±0.7	24.3±2.2	- 7.51±0.15	0.64±0.06	1.01±0.02	15.56±0.04	36.0±0.0

For $\delta^{13}C$ there are significant differences among regions ($F_{3,76}$ = 5.396, P = 0.002) namely between Peniche and Cabo do Mundo, and Peniche and Olhos de Água (Tukey test, P < 0.05)

(Fig. 2A). However no significant differences were found between sites ($F_{2,57}$ = 1.978, P = 0.148) (Fig. 2B).

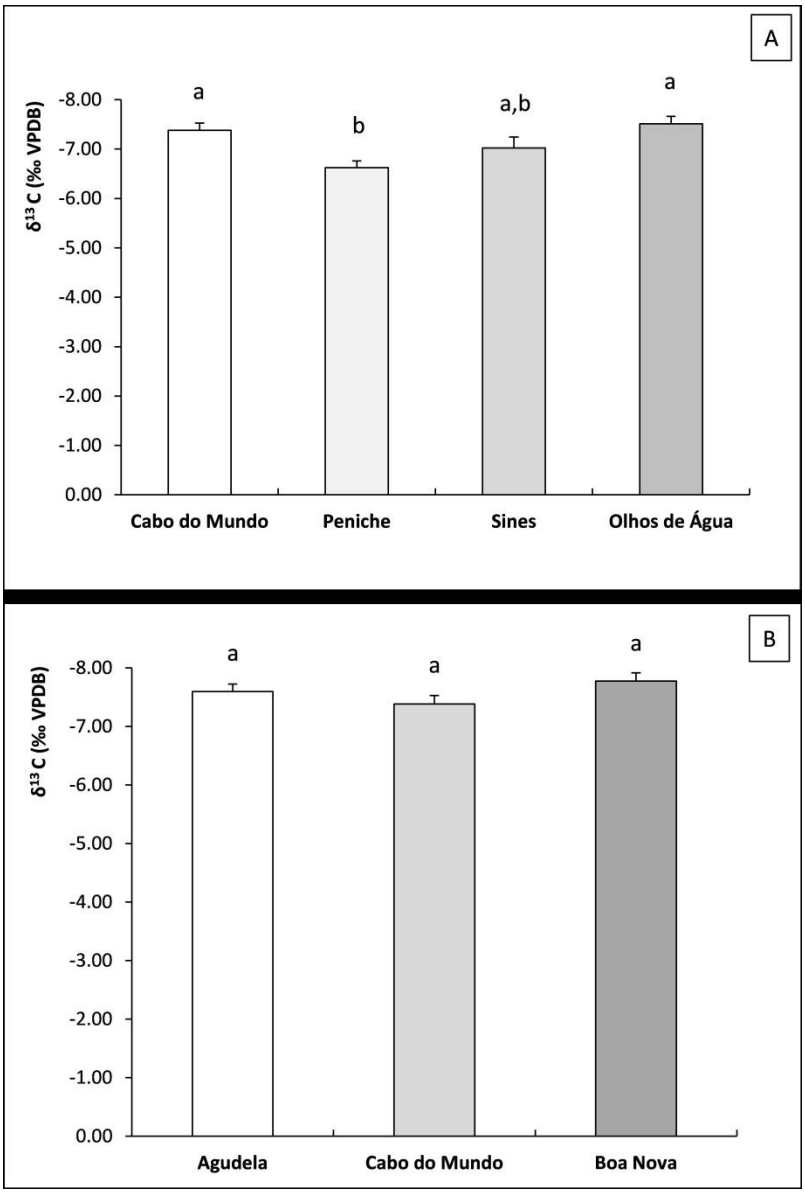

Fig. 2: Carbon isotopic ratios (mean ± SE) recorded in whole otoliths of *Lipophrys pholis* from individuals collected in the four main regions (A) and in the three NW sites (B). The locations marked with the same letter above the error bars are not significantly different for each other (P>0.05)

For δ ^{18}O, there were significant differences between regions ($F_{3,76}$ = 39.852, P = 0.000) (Fig.3A) with the exception of Sines and Olhos de Água (Tukey Test: P>0.05). However between sites no significant differences were observed ($F_{2,57}$ = 0.498, P = 0.610) (Fig. 3B).

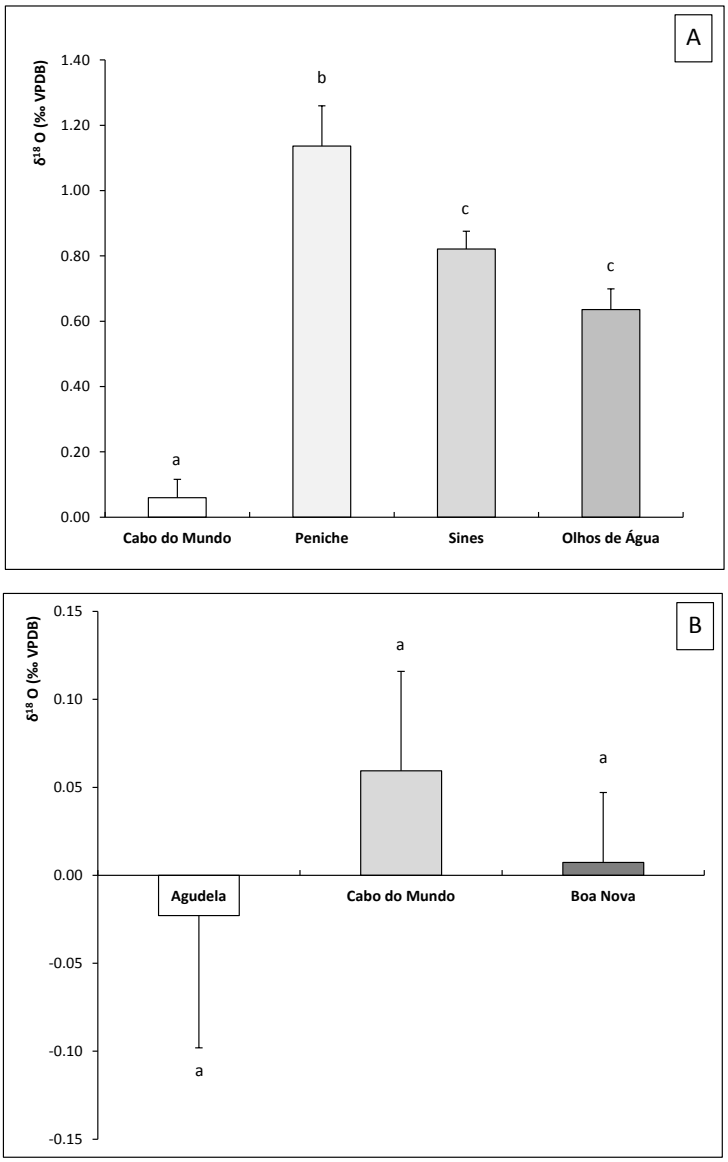

Fig. 3: Oxygen isotopic ratios (mean ± SE) observed in whole otolith of *Lipophrys pholis* from individuals collected in the four main regions (A) and in the three NW sites (B). The location marked with the same letter above the error bars are not significantly different for each other (P>0.05)

For each location both isotopic ratios did not show any clear trend with otolith mass (Figs. 4A and 4B). However for $\delta^{13}C$, otolith mass was significant as co-variate term (ANCOVA, n = 120, P = 0.025) and only 24% of the sum of squares was explained by location (ANCOVA, n = 120, P < 0.05) (Table 2A). For $\delta^{18}O$, 69% of the sum of squares was explained by location (ANCOVA, n = 120, P = 0.000), and otolith mass was not significant (ANCOVA, n = 120, P = 0.275) (Table 2B).

Table 2: ANCOVA for $\delta^{13}C$ (A) and $\delta^{18}O$ (B) values of otoliths

A

Source	DF	SS	MS	F-Ratio	P-Value
Location	5	18.467	3.693	7.567	0.000
Otolith weight	1	2.511	2.511	5.145	0.025
Error	11	55.156	0.488		
Total	17	76.134			

B

Source	DF	SS	MS	F-Ratio	P-Value
Location	5	22.852	4.570	52.743	0.000
Otolith weight	1	0.104	0.104	1.205	0.275
Error	11	9.792	0.087		
Total	17	32.754			

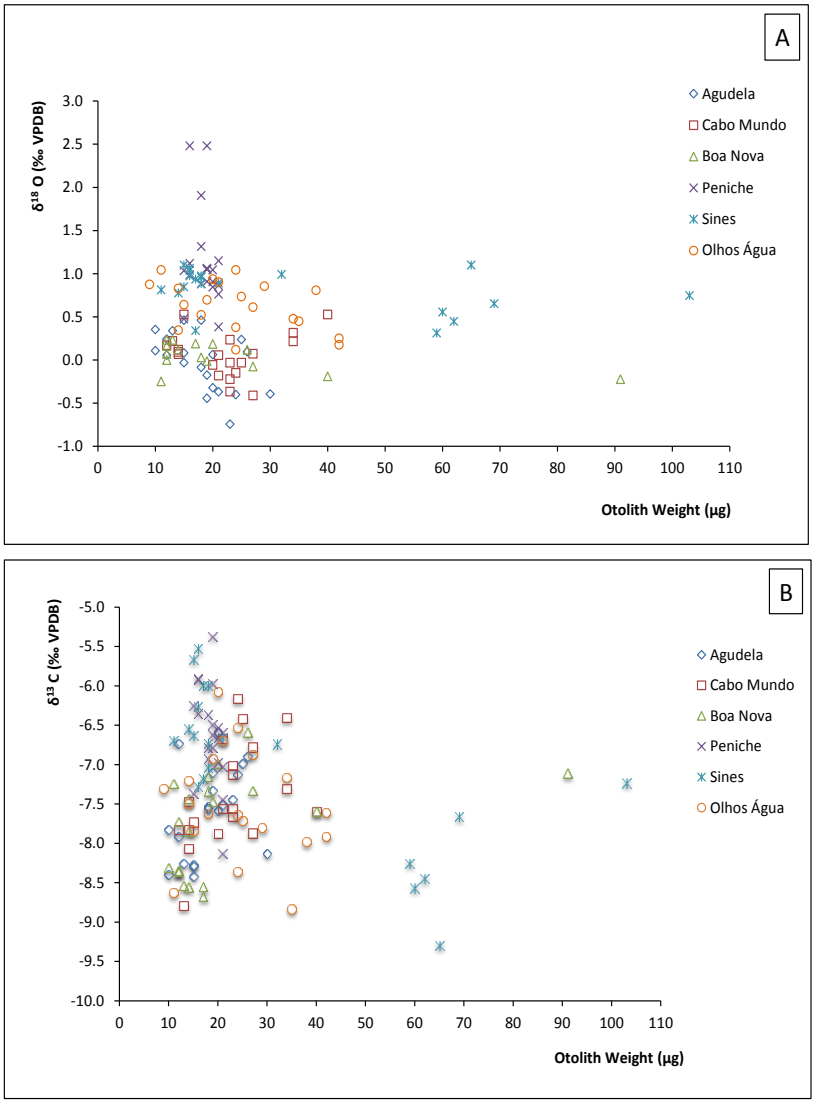

Fig. 4 δ^{18} O (A) and: δ^{13} C (B) otolith signatures versus otoliths mass for all data.

$\delta^{18}O$ values were low but significantly negatively correlated with SST if we exclude the NW sites from the analysis (R^2 = 0.20, N = 60, P < 0.05) (Fig. 5A). However $\delta^{13}C$ values appears to have no significant trends with SST (Fig. 5B).

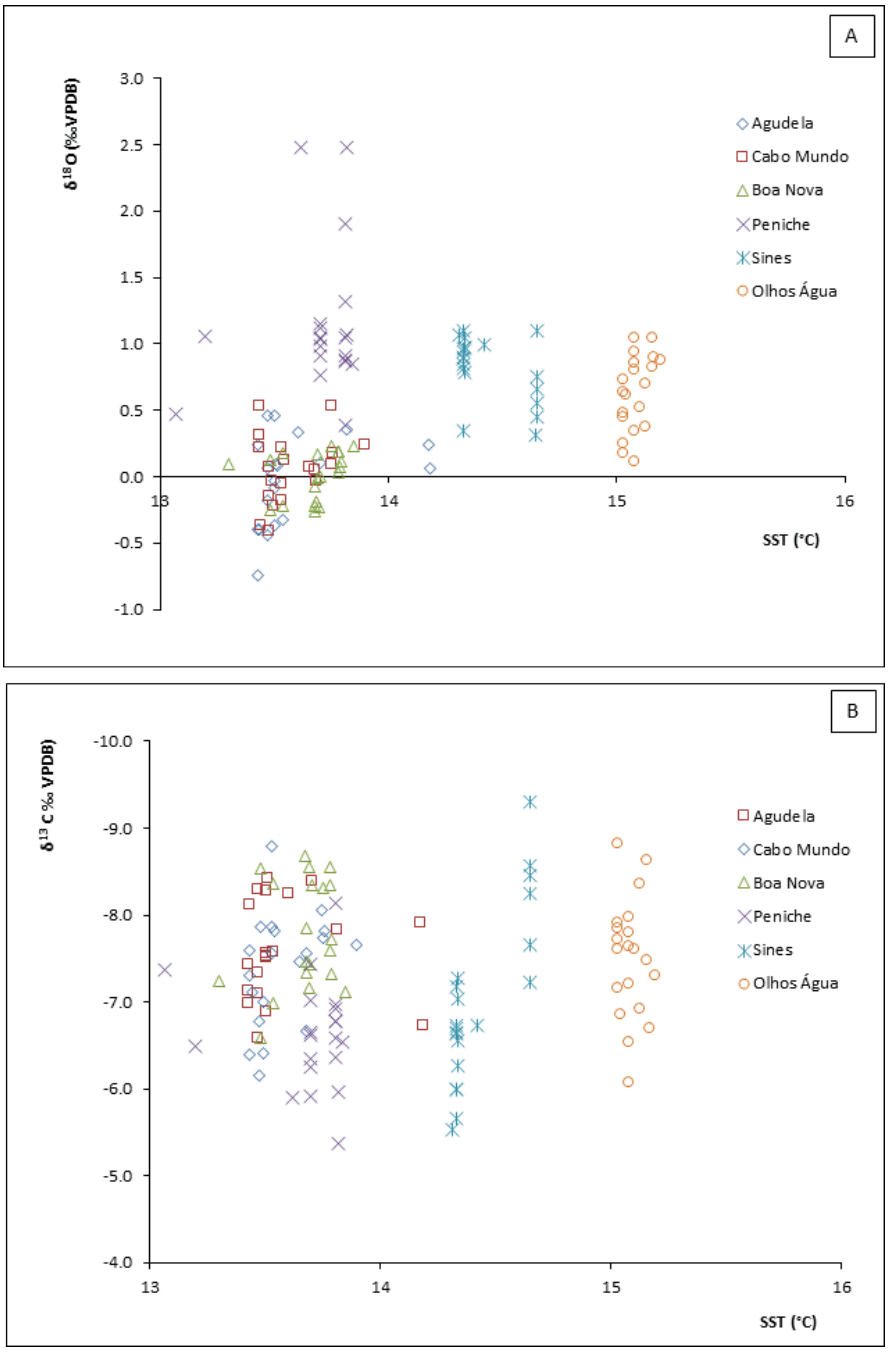

Fig. 5: $\delta^{18}O$ versus mean SST (A) and $\delta^{13}C$ versus mean SST (B) for all data

The mean water isotopic ratios obtained from the six sampling locations ranged from 0.38 to 1.01 VSMOW (Table 1). A positive relationship was found between salinity and oxygen isotopic composition in water at the time samples were collected (Figs. 6A and 6B).

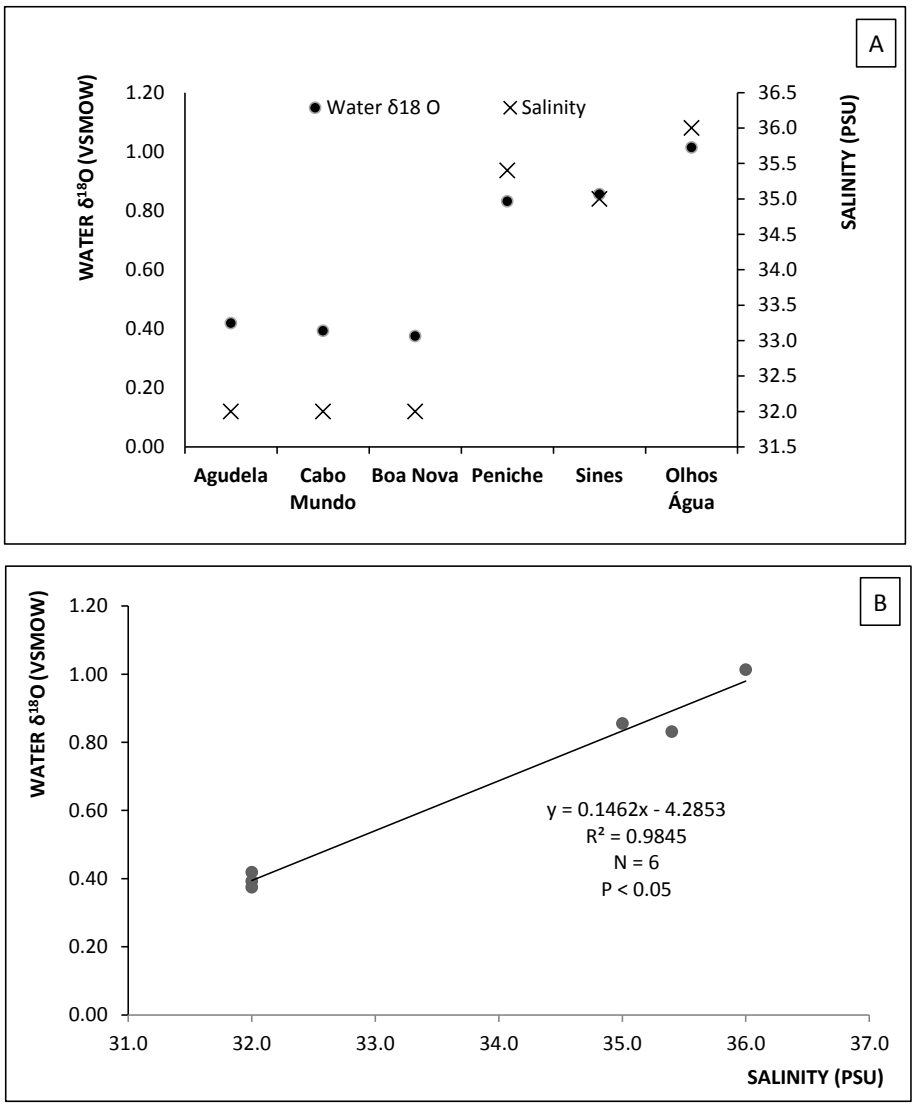

Fig. 6: Variation of the collected water $\delta^{18}O$ and historical recorded sea surface salinities for the sampling locations (A); and linear regression between water $\delta^{18}O$ and sea surface salinities (B).

MANOVA indicated significant differences between regions using the multi-isotopic signatures of the whole otolith (Pillai Trace; $F_{6,152} = 13.911$; $P < 0.05$), but no differences were found between sites (Pillai Trace; $F_{4;114} = 1.332$, $P > 0.05$). All pairwise comparisons between sampling areas were significant (Hotelling's T-square, $P < 0.05$), with the exception of the locations Sines and Olhos de Água (Hotelling's T-square, $P = 0.071$).

The bi-plot using both isotope ratios suggests that the isotopic signatures partially overlap for all regions, namely between Sines and Olhos de Água (Fig. 7A). QDFA based on the whole otolith isotopic composition successfully discriminated four regions, although some overlapping existed between them (Fig. 8A), lowering the classification accuracy rates (85%, 60%, 45% and 45% for Cabo do Mundo, Peniche, Olhos de Água and Sines, respectively) (Table 3A). According to the between group F matrix (df = 2,75) values from QDFA centroids for Sines and Olhos de Água were the closest (2.875) while Cabo do Mundo and Peniche (54.648) were the farthest apart.

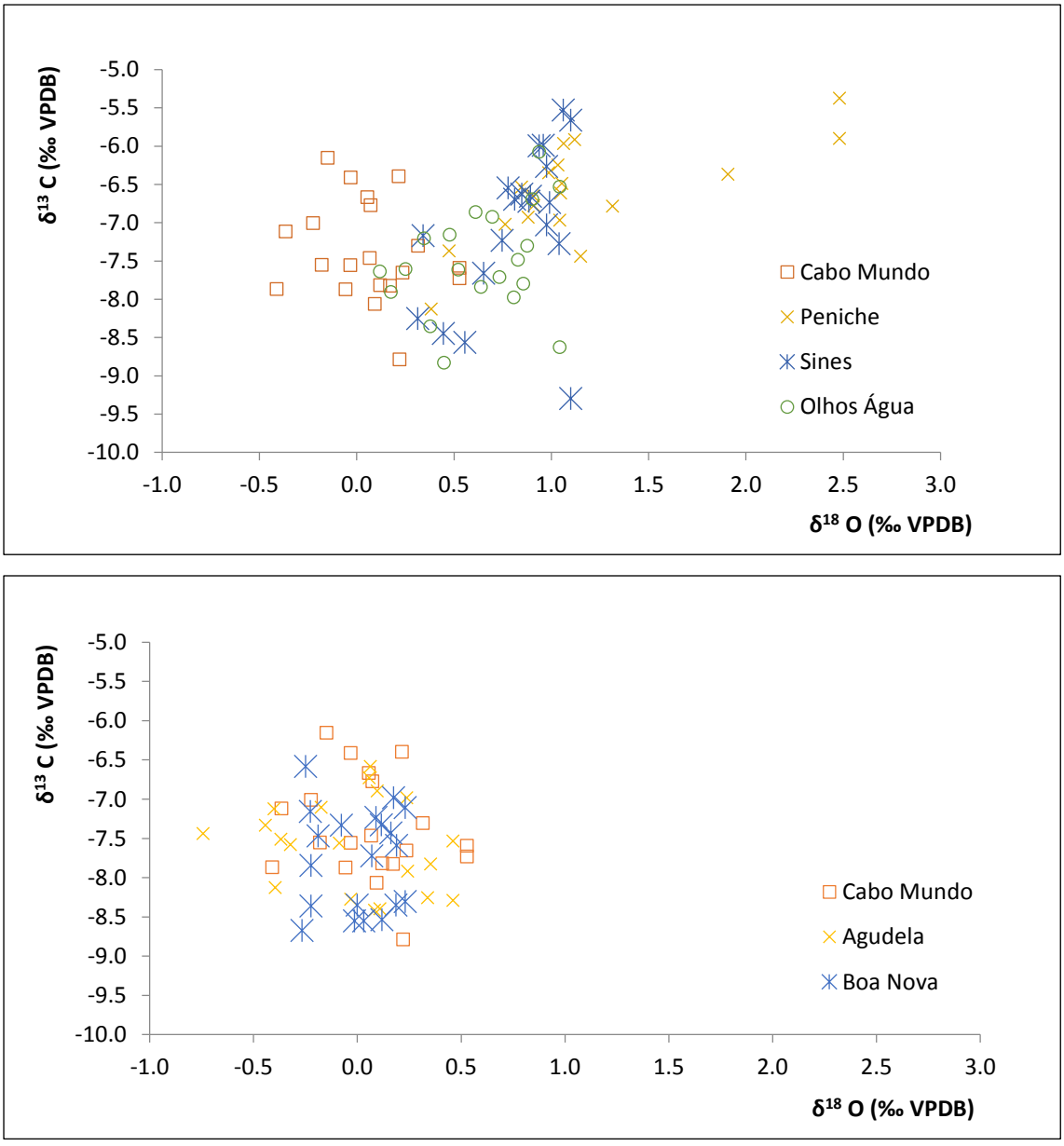

Fig. 7: $\delta^{13}C$ versus $\delta^{18}O$ for sagittal otolith carbonate from *Lipophrys pholis* for the regions (A) and sites (B).

For the comparison between sites, both bi-plot (Fig. 7B) and QFDA (Fig. 8B) registered a significant overlapping of the whole otolith isotopic composition between Agudela, Cabo do Mundo and Boa Nova. Jackknife classification accuracy rates were 50%, 40% and 40% for Boa Nova, Agudela and Cabo do Mundo, respectively (Table 3B). According to the between group F matrix (df = 2,75) values from QDFA centroids for Agudela and Boa Nova were closest (0.433) and Cabo do Mundo and Boa Nova (2.383) are farthest apart.

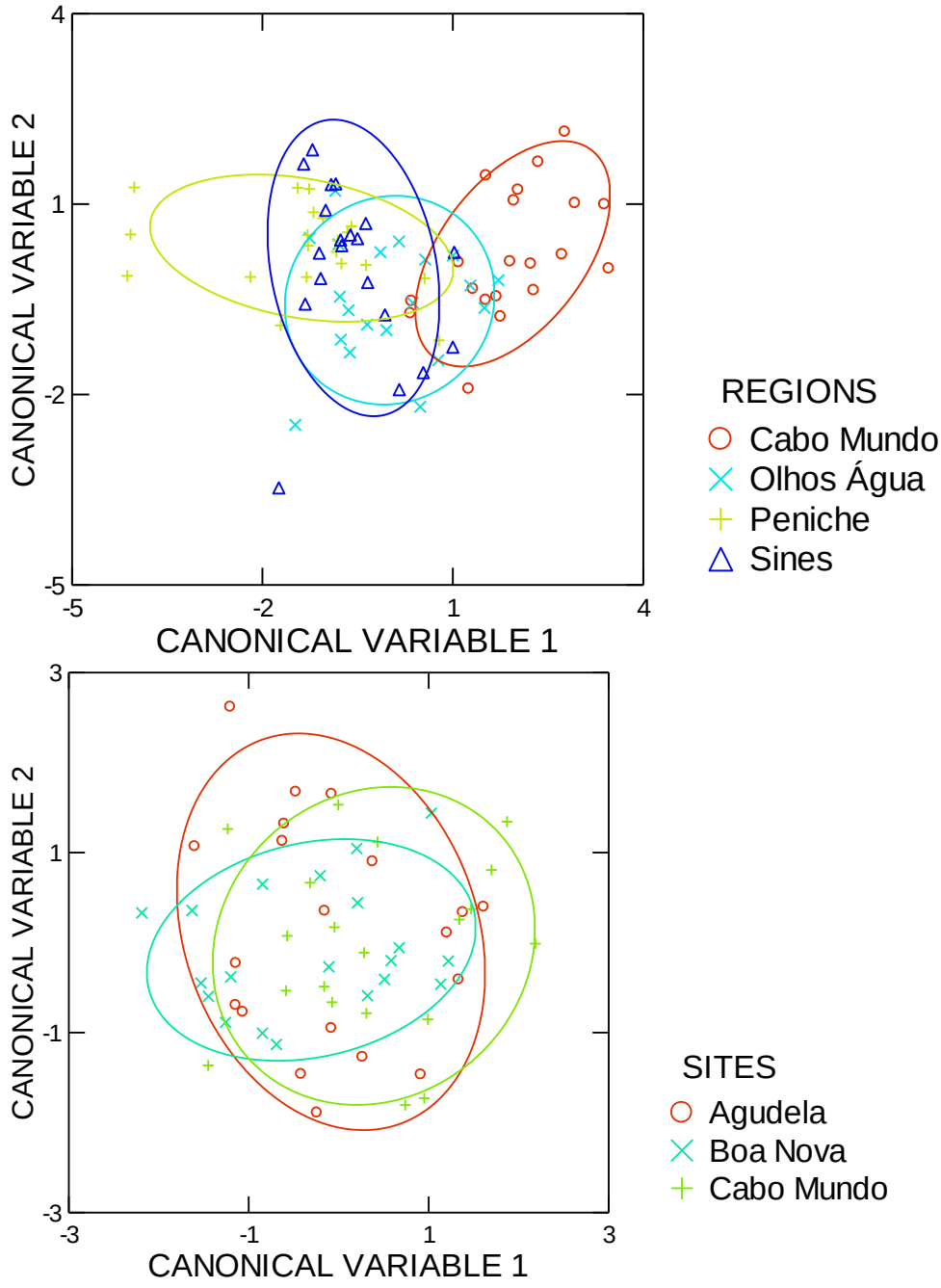

Fig. 8: Canonical variate plots displaying spatial differences in multi-isotopic signatures in whole otoliths of *Lipophrys pholis* from the regions (A) and sites (B) along the Portuguese coast.

Table 3: Jacknife classification matrix of *L. pholis* specimens based on whole otolith isotopic signatures used in QDFA for the sampling regions (A) and sites (B)

A		Predicted Region				
		Cabo do Mundo	Peniche	Sines	Olhos de Água	% Correct
Real Region	Cabo do Mundo	17	0	0	3	85
	Peniche	0	12	5	3	60
	Sines	1	4	9	6	45
	Olhos de Água	4	2	5	9	45
	Total	22	18	19	21	59

B		Predicted Site			
		Agudela	Boa Nova	Cabo do Mundo	% Correct
Real Site	Agudela	8	4	8	40
	Cabo do Mundo	4	8	8	40
	Boa Nova	3	10	7	50
	Total	15	22	23	43

Discussion

Otolith stable isotope ratios have been used to reconstruct environmental temperatures, differentiate among groups, infer metabolic history and reconstruct migration patterns of fish (Campana 1999). In the present study, the isotopic composition of the whole otoliths of *L. pholis* juveniles was assessed at small (sites) and large (regions) scales ranging from three to hundreds of kilometers, respectively. The variation in $\delta^{13}C$ and $\delta^{18}O$ values in *L. pholis* otoliths were within the general range for marine species (-9 to + 1‰ and -2 to +4‰, respectively, for $\delta^{13}C$ and $\delta^{18}O$) (Gerard & Muhling 2010; Correia et al. 2011; Daros et al. 2016). A distinct $\delta^{13}C$ signature for *L. pholis* otoliths was only evident in Peniche which was different from Cabo do Mundo and Olhos de Água. For $\delta^{18}O$, all regions registered significantly different values, except for the two southern regions (Sines and Olhos de Água). However comparison between sites (Agudela, Cabo do Mundo and Boa Nova) did not identify any differences in either $\delta^{13}C$ or $\delta^{18}O$ values.

The source of stable isotopes incorporated into the otolith varies according to the element. Oxygen appears to be incorporated into the otolith with isotopic ratios which are nearly identical to those of the ambient water, although they are also influenced by temperature and

salinity (Campana 1999). The fractionation factor of $\delta^{18}O$ in otoliths is temperature dependent (Thorrold et al. 1997) and the negative linear relationship between water temperature and $\delta^{18}O$ in otoliths has been validated for several species (e.g. Høie et al. 2004; Valle & Herzka 2008; Shiao et al. 2010).

Sea surface temperatures in this study showed the expected latitudinal variation from south to north (Olhos de Água > Sines > Peniche > Cabo do Mundo: 15.6°C > 14.8°C > 13.9°C > 13.7°C). Sea surface salinity values also registered a similar trend, with values of 36.0 > 35.0 > 35.4 > 32.0 psu from south to north respectively. Dissolved $\delta^{18}O$ in water (VSMOW) showed a variation between four main sampling regions (Olhos de Água > Sines > Peniche > Cabo do Mundo: 1.01 > 0.86 > 0.83 > 0.39) whereas otolith $\delta^{18}O$ values were 0.64 in Olhos de Água, 0.82 in Sines, 1.14 in Peniche and 0.06 in Cabo do Mundo.

The hereby observed otolith $\delta^{18}O$ values across the sampled area followed, in general, the expected trend both in relation to water temperature (i.e. higher oxygen isotopic ratios at areas with lower SST values), water isotopic composition and salinity. However, individuals collected in the NW region (Cabo do Mundo) exhibited unexpectedly low otolith $\delta^{18}O$ values with regard to their SST probably because it is the only sampling location near a major source of freshwater run-off (Correia et al. 2011). The Douro river is responsible for an input of freshwater into the Portuguese inner shelf, with an average freshwater discharge of $488 m^3 s^{-1}$ (Vieira & Bordalo 2000). The surrounding area is a known to have a low-salinity lens (Western Iberian Buoyant Plume, WIBP) formed by river discharge and continental run-off extending along the shelf off Northwest Iberia (Otero et al. 2008). Moreover salinity is also commonly used as a proxy for water $\delta^{18}O$ values through the fractional amount of runoff-sourced freshwater namely in the shallow coastal, and the $\delta^{18}O$ profiles otoliths can reflect seasonal freshwater input (Matta et al. 2013). This suggests that the lower $\delta^{18}O$ values found in individuals from NW region are probably related with the nearby environmental salinity (Elsdon & Gillanders 2002).

For $\delta^{13}C$, 10% to 30% of otolith carbon may be derived from metabolic sources suggesting a dietary origin. The remainder of the otolith isotopic carbon composition would presumably come from dissolved inorganic carbon (DIC) in the water (Campana 1999). A weak but significant relationship between $\delta^{13}C$ and otolith mass (which is considered to be proportional to fish age) was found in this study. This could be the result of an ontogenetic change in the feeding regime related to a known shift in the pattern of microhabitat occupation of juveniles as they grow (Faria & Almada, 2001a). Furthermore a unique $\delta^{13}C$ signature of *L. pholis* was only partially evident for in Peniche. The differences in $\delta^{13}C$ observed among regions may indicate that *L. pholis* had a different food source or diet. However, between sites, *L. pholis* seems to have similar food sources. However the effect of the fish growth rate and metabolism on the

$\delta^{13}C$ otolith signatures cannot be discarded to explain this findings (Thorrold et al. 1997; Hoie et al. 2003; Geffen 2012). Unfortunately no seawater carbon isotopic data is available from the study region to draw further considerations.

Fish from different regions were not discriminated very well based and the maximum reclassification success reported was 85%, otherwise 60% or lower. The bi-plot and QFDA allowed us however to identify three main groups: Cabo do Mundo, Peniche and Sines/Olhos de Água. In fact if a QDFA (not show here) is run considering the SW and S regions as a single group, the reclassification rates became significantly higher: 85% (Cabo do Mundo), 80% (Peniche) and 60% (Sines/Olhos de Água). This data suggests a limited spatial movement of *L. pholis* during their juvenile phase, at least for the northern and central regions. This also suggests that *L. pholis* shows some kind of residency fidelity to the growing areas, with exception of the southern locations. However, a significant overlap in isotopic signatures and low maximum reclassification success rates (50% or less) were observed between sites suggesting some type of larval retention mechanism and/or significant juvenile movements at a small scale distance (c. 3 km). The distribution pattern observed here might be related to both coastal spawning strategy and low dispersal due to the non-pelagic nature of their eggs already reported. It is well-known that *L. pholis* have benthic or demersal eggs, which are probably not affected by advective processes until they hatch, thus reducing the chances of being transported offshore (Azeiteiro et al. 2006). Furthermore, *L. pholis* larvae could resist oceanic dispersion by currents due to their active swimming abilities (Hickford & Schiel, 2003; Fisher 2005; Leis et al. 2006).

This study showed that otolith stable isotopic differences found at large spatial scales, at least for the occidental northern and central regions, indicate that *L. pholis* experience different environmental conditions during their juvenile phase and/or limited movements between regions. Stable isotopic ratios also suggest high connectivity and/or a larval retention at small spatial scales. More studies are however need to understand if *L. pholis* follows a metapopulational structure or a self-recruitment process.

Acknowledgments

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4.2 Use of otolith elemental signatures as natural tags to evaluate the larval dispersion, coastal recruitment, habitat connectivity and population structure of *Lipophrys pholis*

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Abstract

Otolith's geochemical signatures can be used as a tool to identify the fish source origin once they have the capacity to reflect the water chemistry of the environment here fish lived. *L. pholis* embryos and recruits from the same cohort were collected in 2013 from 17 sites within 3 main regions of the Portuguese coast (NW, CW and S regions). Laser ablation inductively coupled plasma mass spectrometry was used to measure the concentration of 7 informative elemental ratios in the otolith's core. Molar ratios of Li/Ca, Mg/Ca,P/Ca, S/Ca, Mn/Ca, Sr/Ca and Ba/Ca show that natal chemical signatures are somewhat spatially specific for *L. pholis*. However multi-elemental signatures are highly different between sub-regions and/or sites. The population connectivity matrix identified diferente dispersal pathways for *L.pholis* embryos. Cabo do Mundo was an important source population for NW region. For the CW region, Peniche was the site that most contribute as source population for Estremadura Norte sub

region; Praia das Maças was the site that contribute most for Estemadura Sul population; and Alpertuche was the greatest contribution for Arrábida sub region source. SW registered similar values for self-recruitment for both Odeceixe and Sines sites. 20% to 53 % of early juveniles may be returning to their natal population (self-recruitment), but others came from other areas mainly from southern locations. It also means that fish larvae disperse away from their natal population so that local populations operate as 'open' systems driven by recruitment of larvae from other sub-populations suggestion a metapopulation structure. Furthermore unexpectedly the SW is the main contributor for all the main regions and larvae are probably driven by the northward flow of the Portuguese Coastal Counter Current during winter suggesting that long-distance dispersal is the norm for *L. pholis* fish populations. However this data should be look with careful and further studies should assess if this pattern persist in the following years.

Key words: Blenniidae; otolith fingerprinting; larval dispersion; habitat connectivity; population structure

Introduction

Lipophrys pholis (Linnaeus 1758), commonly referred to as shanny, has a wide geographical distribution from Norway to northern Morocco (Zander, 1986). However it is located at much higher latitude than other European blennids, indicating that is a species adapted to colder waters (Almada et al., 1990). *L. pholis* is an intertidal species highly abundant along the Portuguese coast (Almada et al., 1990). In Portugal the reproductive season occurs from early Autumn to middle Spring (October/November to May) (Faria et al., 1996) at a small size and very young age (Carvalho et al, 2017) According captive experiments embryonic development lasts 16 days at 17º (Faria et al., 2012). After hatching the pelagic larvae disperse to the coastal area and individuals apparently return to a particular set of rock tide pools, two to three months later, in early winter to settle (Faria et al., 1996). A mitochondrial DNA study of *L. pholis* found no significant population genetic structure along the Portuguese coast, attributing the genetic homogeneity of this species to an efficient gene flow as result of the oceanic dispersal of the planktonic larvae (Francisco et al., 2006). An earlier study that used oxygen and carbon stable isotopes recorded in whole otoliths of individuals collected in some Portuguese rocky beaches suggested that *L. pholis* experience different environmental conditions during their juvenile phase and/or limited movements

between regions at large spatial scales, but at small scales data showed considerable habitat connectivity and/or larval retention (Carvalho, unpublished data).

Otolith chemistry analysis is an innovative approach that can complement other stock discrimination techniques, such as the traditional tag/recapture and genotypic approaches, as an effective method to assess stock structure, migration patterns and connectivity between juvenile/adult populations and spawning/nursery sources (Campana et al., 2000; Elsdon & Gillanders., 2004; Correia et al., 2011).Natural tags, such as the geochemical composition of calcified structures of marine organisms, are increasingly being employed as a strategic tool in marine research (Gomes et al*.,* 2016). These been applied to determine natal signatures and dispersal patterns, using crustacean embryos and larvae (DiBacco & Levin 2000, Carson 2010), fish otoliths (e.g. Swearer et al., 1999; Correia et al., 2014), larval mollusk statoliths (Zacherl 2005) and shells (e.g. (Becker et al., 2007, Carson 2010, Gomes et al., 2016). This method requires not only the existence of location-specific chemical signatures at the site of origin but also the maintenance of these 'natal tags' after settlement (Thorrold et al., 2007).

This study aims to analyze the otolith core of *L. pholis* recruits and match them to the embryonic (natal) otolith signatures from known locations, which will ultimately allow us to assign the recruits to their putative source population. Such valuable empirical data on larval sources can be used to validate biophysical models of larval dispersal, coastal recruitment and habitat connectivity, which can unravel the underlying fish population structure mechanisms of *L. pholis*.

MATERIAL AND METHODS

Area Description

The west coast of the Iberian Peninsula is located on the northernmost limit of the Eastern North Atlantic Upwelling Region, running for about 700 km along a north-south direction. At the north and south extremes, near Capes Finisterre and S. Vicente, the coast veers east and extends for a few hundreds of kilometers (Nolasco et al., 2013). Several estuarine systems and rias occur along the west Iberian coast, while stretches of sandy shores alternate with rocky shores on scales of kilometers to a few tens of kilometers. This geological setup provides a conceptually interesting environment to test population connectivity in coastal species (Nolasco et al., 2013), because it effectively demarcates local populations along an approximately linear coast, with the exception of a few coastal islands and shallow bays. Circulation off the west Iberian coast (Peliz et al. 2005, Relvas et al., 2007) is mostly controlled

by the interaction of the Iberian Poleward Current (IPC) with the upwelling/downwelling circulation driven by along-shore winds and with the Western Iberian Buoyant Plume (WIBP).

The study area was divided in 3 main regions: NW (North-west), CW (Central- west) and SW (South-west) regions which was further sub-divided mainly for statistical analysis purposes in sub-regions and sites (Table 1) (Fig. 1).

Table 1. Main Regions: NW (North West); CW (Central West): and SW (South West region), Sub region and sites of *L. pholis* sampling.

Region	Sub region	Site
NW	Viana Do castelo	Póvoa do Varzim
	Alto Douro	Cabo dom Mundo
	Baixo Mondego	Figueira da Foz
CW	Estremadura Norte	Miradouro da Arrinhada
		Berlengas
		Peniche
	Estremadura Sul	Foz do Sisandro
		Samarra
		Maçãs
	Arrábida	Lagosteiros
		Cova da Mijona
		Alpertuche
	Cascais	Cabo Raso
		Bafureira
SW	Santiago do Cacém	Sines
	Costa Vicentina Norte	Milfontes

Biological Sampling

Embryos samples were collected from *L. pholis* nests along the Portuguese coast during March 2013. Sampling sites were selected based on the knowledge of the oceanic currents, considering the possible larval dispersal and presence of rocky shore habitats. Therefore, 17 sites (rocky beaches) along the occidental Portuguese coast (NW, CW and SW) were selected for the sampling of the embryos (Fig. 1)

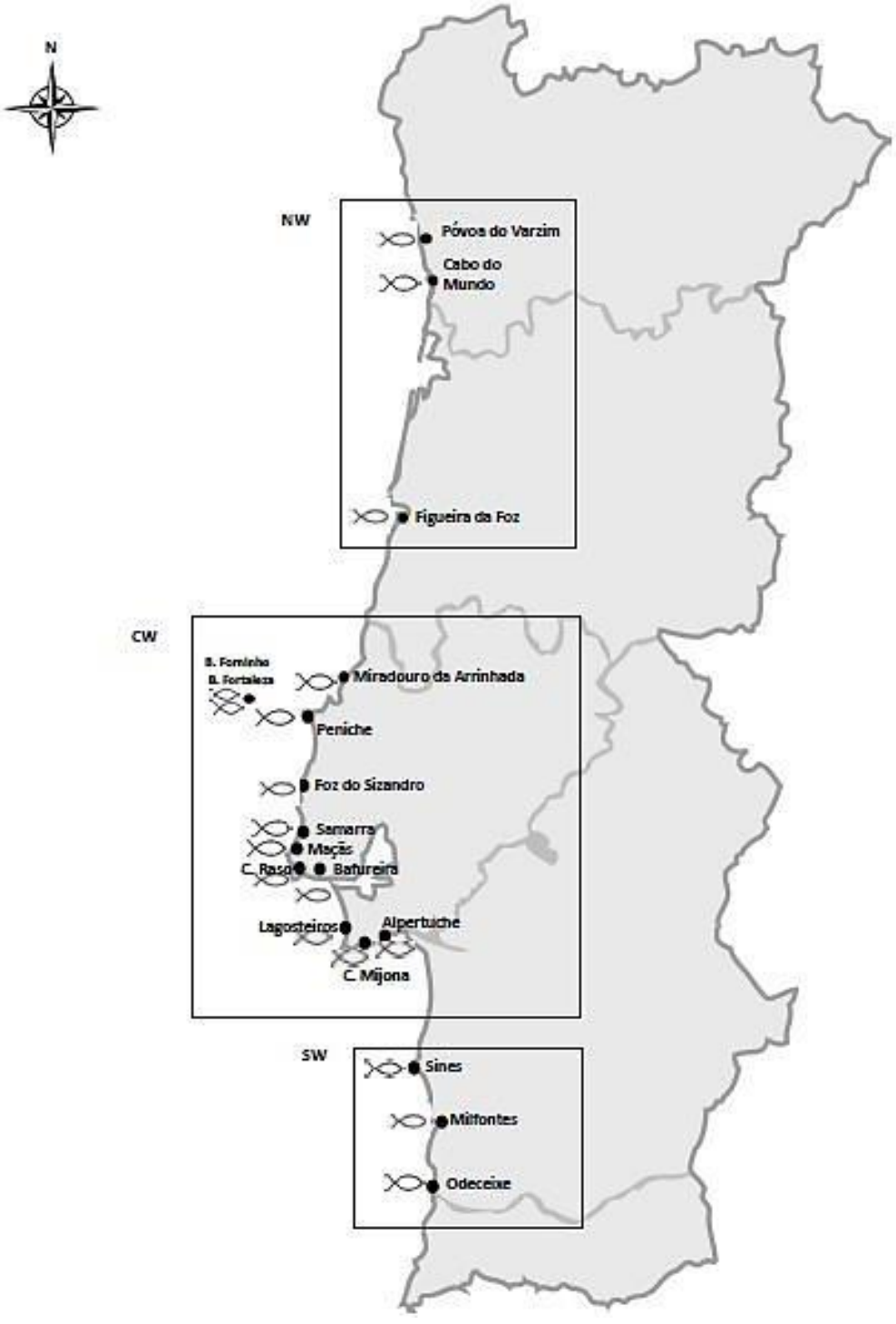

Fig. 1. Embryos (●) and juvenile sampling sites ( ) of *Liphorys pholis* for NW, CW and SW regions

Lipophrys pholis eggs in the latest development stage (stage D: Faria et al. 2002) were collected from three nearby nests (replicas) at each site. Since this species has parental care, the presence or absence of the *L. pholis* male in the nest was recorded to ensure the species provenance of the eggs (Almada & Santos 1995). The eggs were stored in decontaminated vials, filled with local sea water, transported to the laboratory and frozen for future analysis.

Samples of recruits from the same cohort were collected in April and May 2013, approximately. 15 individuals per site (5 per tide pool – replicates) less than 30 mm total length were stored in decontaminated vials, filled with local sea water, transported to the laboratory and frozen for future analysis. Total length (TL, nearest 0.1 cm) was measured in all individuals.

Otolith Preparation

Sagittal otoliths of embryos and recruits were extracted under an Olympus SZX10 coupled with Olympus DP25 camera at x 15 magnification. To avoid contamination a strict protocol was followed for both embryos and recruits. During the extraction, cleaning and mounting standard milli-Q water and certified trace metals reagents were used. For the preparation of the samples only plastic material and titanium/tungsten metal instruments were used. All plastic material was leached in an acid solution (1N HCl) for 48 hours, rinsed in milli-Q water, dried in a laminar flow chamber and stored in sealed zip bags. The other material and instruments that were in contact with the samples were acid leached in an 0.01 N HCL solution and rinsed with milli-Q water.

One sagittal otolith was extracted from each embryo/recruit using a fine tungsten dissecting pin. For transferring the otoliths from one droplet of liquid to another we used a superfine synthetic paintbrush (type 10/0). To remove any residual organic tissue, the otolith was placed in a cleaning solution of 15% H_2O_2 for 10 minutes (Hydrogen peroxide 30% Merck Suprapur) buffered with 0.1N NaOH (Sodium Hydroxide monohydrate 99.99% Merck Suprapur). Immediately after, the embryos otoliths were rinsed 3 times in milli-Q water, placed on a gridded microscope slide coated beforehand with Buehler's Epothin Epoxy resin and mounted in a small amount of the same resin.

Recruits otoliths, after decontamination, were glued with Buehler's Epothin Epoxy resin in small 1.5 mm discs made of the same resin. After the resin has dried out, the otoliths in the disc were grounded following the sagittal plane using Apex Diamond grinding discs of 6, 3 and 0.5 microns (Buehler). During this procedure, frequent checks were made using light microscopy (Olympus, CX41; www. olympus. com/) until the core was revealed. Thereafter

otoliths were polished with 3, 1 and 0.25 microns' diamond polishing compound (Buehler). When all the samples were finished, they were randomly mounted in glass slides previously identified, and a map of the slide was made to identify the samples after the reading of the elemental fingerprints.

Given the aforementioned objective of this study, which prompted the polishing of the recruit's otolith in order to expose the core region, here we only present the sample elemental concentration of the core region for both embryos and recruits.

Otolith Natal Fingerprints

Trace element concentrations of *L. pholis* otoliths were determined using an Agilent 7700 Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) coupled to a HelEx (Laurin Technic and the Australian National University) laser ablation (LA) system with a 193 nm Compex 110 (Lambda Physik) excimer laser.

Blocks of 18 samples each were randomly run to avoid possible bias due to short-term instrument drift. Each block of samples was bracketed by runs of 610 and 612 NIST (National Institute of Standards and Technology) glass standards and a matrix-matched consistency standard MACS-3 USGS (US Geological Survey) for estimating external analytical precision (% relative standard deviation, RSD). Preceding each standard and sample analysis a 30 seconds black was acquired to correct for background noise to estimate the limits of detection of the method. The average limits of detection were calculated from the individual calibration curves using the three sigma criteria and were (Element:Ca) (in µmol element mol $^{-1}$): ^{7}Li (6.9), ^{55}Mn (4.1), ^{138}Ba (0.035), Pb (0.051), ^{66}Cu (0.601), Zn (1.396), ^{11}B (0.035) and ^{39}K (0.119); and in (mmol element mol $^{-1}$): ^{24}Mg (0.005),^{31}P(0.26) ^{34}S(0.76) ^{88}Sr (0.001).

Both embryo and recruit otolith microchemical composition were analyzed individually using a beam diameter of 32 µm, a laser energy of 60 mJ and a laser repetition rate of 5 Hz. Otoliths were analyzed along its transverse axis, from the edge and through the core. The elements used were these that were consistently above the LOD: ^{7}Li, ^{24}Mg, ^{31}P, ^{34}S, ^{55}Mn, ^{88}Sr, and ^{138}Ba.

We first filtered the data to eliminate any peaks defined as a single scan value greater than two times the median of three adjacent scans. Data were smoothed using a running average of three scans to reduce noise present in the data due to analytical imprecision. The blanks were then subtracted from standards and sample intensities to obtain net intensities. We only considered the readings that had at least 200 000 counts of Ca, since the majority of elements

were below the detection limit of the method for samples with lower yields. The concentration of each element was standardized to molar ratios relative to calcium, to account for differences in the amount of ablated material.

Statistical analysis

Prior to statistical analyses, all data were transformed [Log(x+1)] in order to meet the normality and homogeneity.

A Nested ANOVA was used to explore individual elemental fingerprint differences with two fixed factors: region and sub-regions (sites) nested within region. This allowed us to identify the elements which showed significant differences in concentrations at these different spatial scales. If significant differences were found, this was followed by a Tukey post hoc test.

MANOVA was used to test for spatial differences in otolith multi-elemental signatures. For the MANOVA, we reported the approximate F-ratio statistic for the most robust test of multivariate statistics (Pillai's trace).

A Linear Discriminant Function Analyses (LDFA) in stepwise mode was used to visualize spatial differences of natal fingerprints of embryos and to examine the re-classification accuracy success of embryos to this original location. Cross-validations were performed by using the jackknife ("leave one out") procedure (Correia et al 2014).

Additionally, a Maximum Likelihood Analysis (MLA) using the embryos core otolith chemistry data as baselines was used to determine the contributions of regions/sites to the collected recruits from the same cohort. The HISEA software was used to conduct the MLA (Millar, 1990). For each cohort, the simulation mode with 1000 simulations was initially used to estimate the variability of the estimator (i.e. baseline data). Bootstrapping (1000 re-samplings) of the baseline and mixed sample data was used to estimate the mean and standard deviations of the proportions of older fish originating from each spawning area (Hamer et al., 2011).

Statistical analyses were performed using Systat (version 12.0).Results are presented as means ± standard errors (SE). The statistical level of significance (α) for all tests was 0.05.

Results

Embryos

Univariate ANOVAs comparing elemental ratios to calcium for the 7 elements used to discriminate *L.pholis* embryos among regions and sub regions resulted in significant differences in all elements, but only for sub-regions (Table 2).

Table 2. Results from univariate ANOVAs of the effect of main region and sub region on trace element concentrations in the embryonic otoliths of *Lipophrys pholis*. **Bold** values indicate significant effects at the 5% of significance level.

Element	Source	df	SS	MS	*F*	p
$^{7}Li:^{43}Ca$	RE	2	$8.01E^{-02}$	$4.01E^{-02}$	3.3913	0.24
	SU(RE)	7	0.42441	$6.06E^{-02}$	5.1331	**0.001**
	Res	203	2.3977	$1.18E^{-02}$		
	Total	212	2.8703			
$^{24}Mg:^{43}Ca$	RE	2	0.45077	0.22538	10.213	0.166
	SU(RE)	7	2.2247	0.31782	14.402	**0.001**
	Res	203	4.4798	$2.07E^{-03}$		
	Total	212	7.9493			
$^{31}P:^{43}Ca$	RE	2	$1.44E^{-02}$	$7.22E^{-03}$	0.31186	0.91
	SU(RE)	7	0.35773	$5.11E^{-02}$	2.207	**0.007**
	Res	203	4.7005	$2.32E^{-02}$		
	Total	212	5.0706			
$^{34}S^{43}Ca$	RE	2	$7.56E^{-03}$	$3.78E^{-03}$	1.1577	0.754
	SU(RE)	7	0.18139	$2.59E^{-02}$	7.9366	**0.001**
	Res	203	0.66278	$3.26E^{-03}$		
	Total	212	0.84462			
$^{55}Mn:^{43}Ca$	RE	2	1.9132	0.95659	2.3745	0.351
	SU(RE)	7	9.1083	1.3012	3.2299	**0.001**
	Res	203	81.781	0.40286		
	Total	212	92.618			
$^{88}Sr:^{43}Ca$	RE	2	$8.37E^{-02}$	$4.18E^{-02}$	8.4142	0.078
	SU(RE)	7	$9.98E^{-02}$	$1.43E^{-02}$	2.8689	**0.002**
	Res	203	1.0092	$4.97E^{-03}$		
	Total	212	1.2112			
$^{138}Ba:^{43}Ca$	RE	2	6.1228	3.0614	12.824	0.452
	SU(RE)	7	14.702	2.1003	8.7982	**0.001**
	Res	203	48.46	0.23872		
	Total	212	70.085			

Single elemental composition of cores showed no significant differences among the three main regions (ANOVA, P > 0.05;

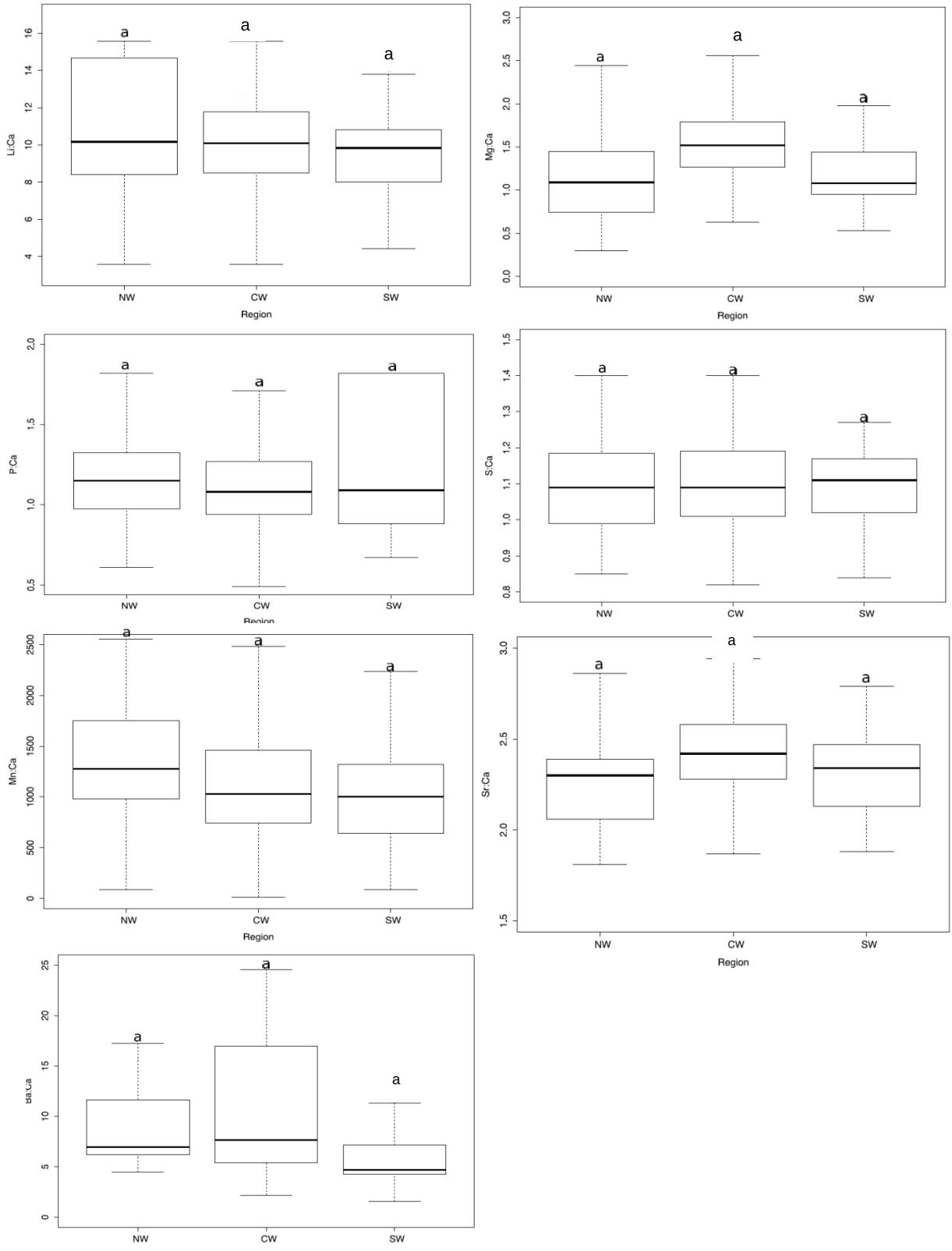

Fig. 2. Molar elemental concentrations (box-plots) from core's otoliths of embryos between the three main regions. The locations marked with the same letter above the error bars are not significantly different concerning the elemental concentrations (P > 0.05). Ratios are given in μmol element mol^{-1} calcium for Li, Mn and Ba. For Mg, P, S, and Sr ratios are given in mmol element mol^{-1}

Single elemental composition of cores showed significant differences among the three NW sub regions (ANOVA, P < 0.05) for all element/Ca ratios except Sr and Mn (ANOVA, P> 0.05)

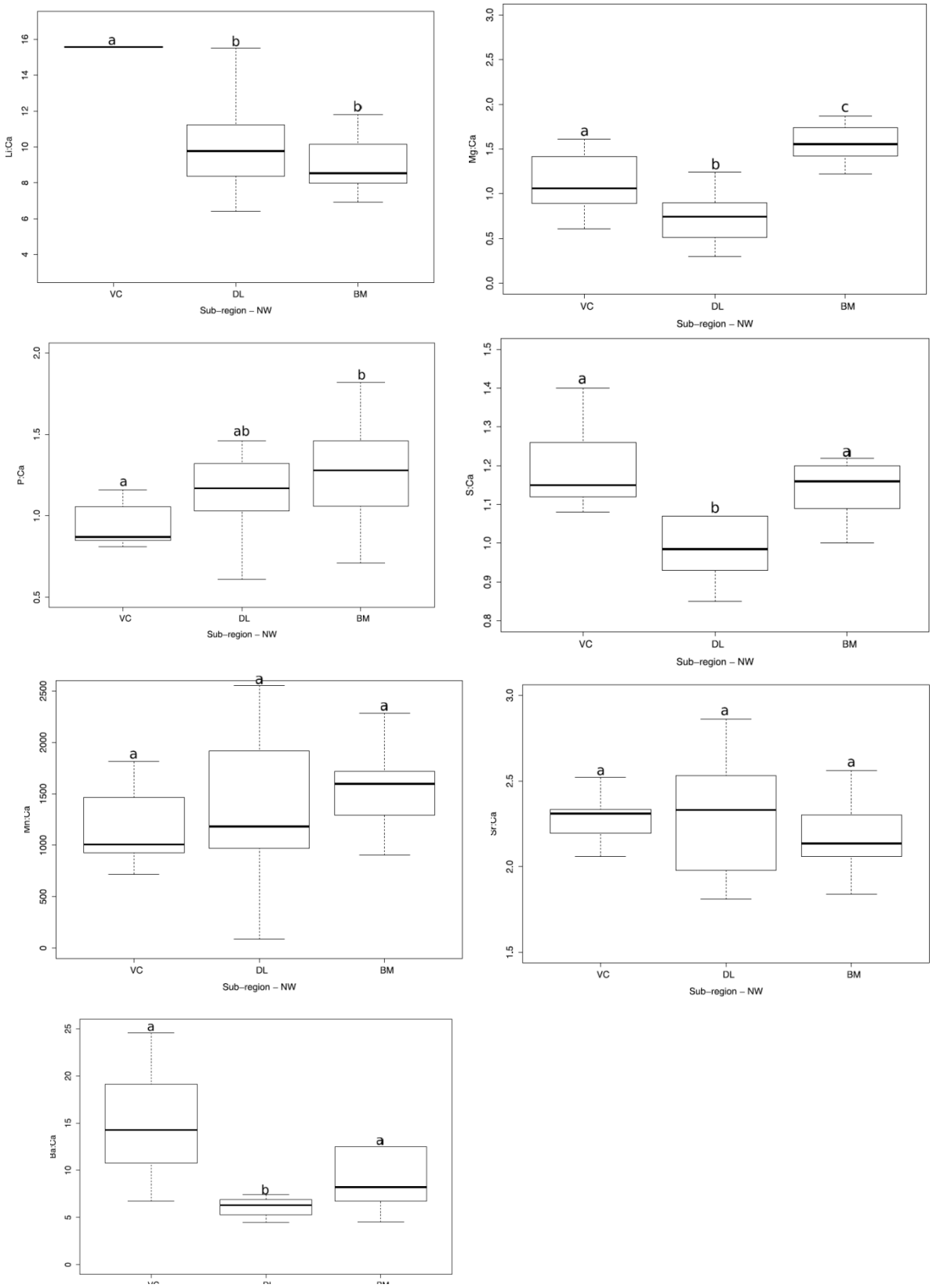

Fig. 3. Molar elemental concentrations (box-plots) from core's otoliths of embryos between NW sub-regions (sites). The locations marked with the same letter above the error bars are not significantly different concerning the elemental concentrations (P > 0.05). Ratios are given in µmol element mol^{-1} calcium for Li, Mn and Ba. For Mg, P, S, and Sr ratios are given in mmol element mol^{-1}

Furthermore, for NW region, MANOVA indicated the existence of significance differences in the multi–element signatures of the otolith cores (Pillai trace, $F_{10,50}$=18.722, P< 0.05) Furthemore, LDFA showed three distinct groups without any overlapping (Fig 4).

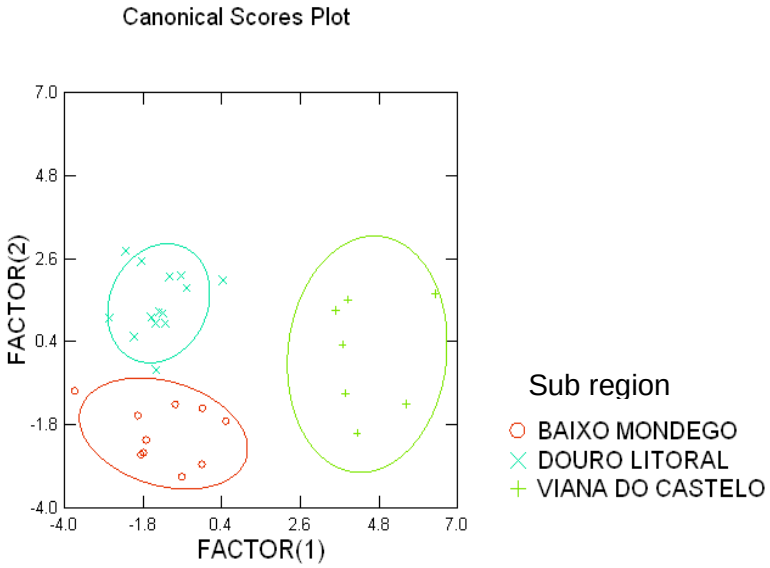

Fig. 4. Canonical variate plots displaying spatial differences in elements signatures in core otoliths of *Lipophrys pholis* embryos from the NW sub region

Jackknifed classification accuracy was very high showing an overall mean of 94% for all embryos of NW sub region region (Table 3).

Table 3. Jacknife classification matrix of *L. pholis* embryos based on core otolith elements used in LDFA in NW sub regions.

		Predicted Region			
		Viana do Castelo	Douro Litoral	Baixo Mondego	% Correct
Real region	Viana do Castelo	7	0	0	100
	Douro Litoral	0	13	1	93
	Baixo Mondego	0	1	9	90
	Total	7	14	10	94

Single elemental composition of cores showed significant differences among the four CW sub regions (ANOVA, $P < 0.05$) for all element/Ca ratios.

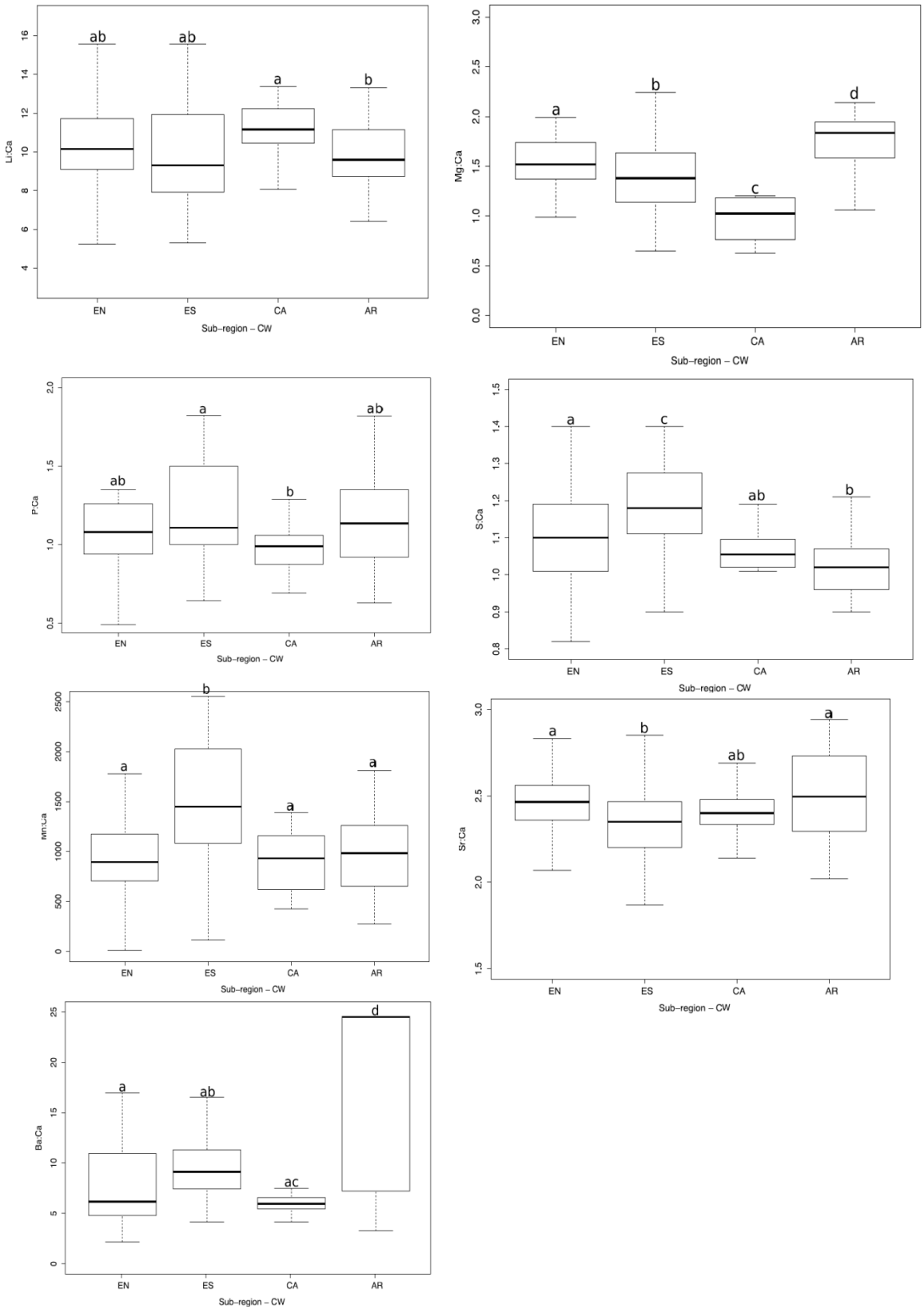

Fig. 5. Molar elemental concentrations (box-plots) from core's otoliths of embryos between CW sub regions. The locations marked with the same letter above the error bars are not significantly different concerning the elemental concentrations ($P > 0.05$). Ratios are given in μmol element mol^{-1} calcium for Li, Mn and Ba. For Mg, P, S, and Sr ratios are given in mmol element mol^{-1}.

For CW region MANOVA indicated the existence of significance differences in the multi – element signatures of the otolith cores (Pillai trace, $F_{21,372}$=8.240, P<0.05). LFDA however showed some overlapping between sub-regions. (Fig. 6)

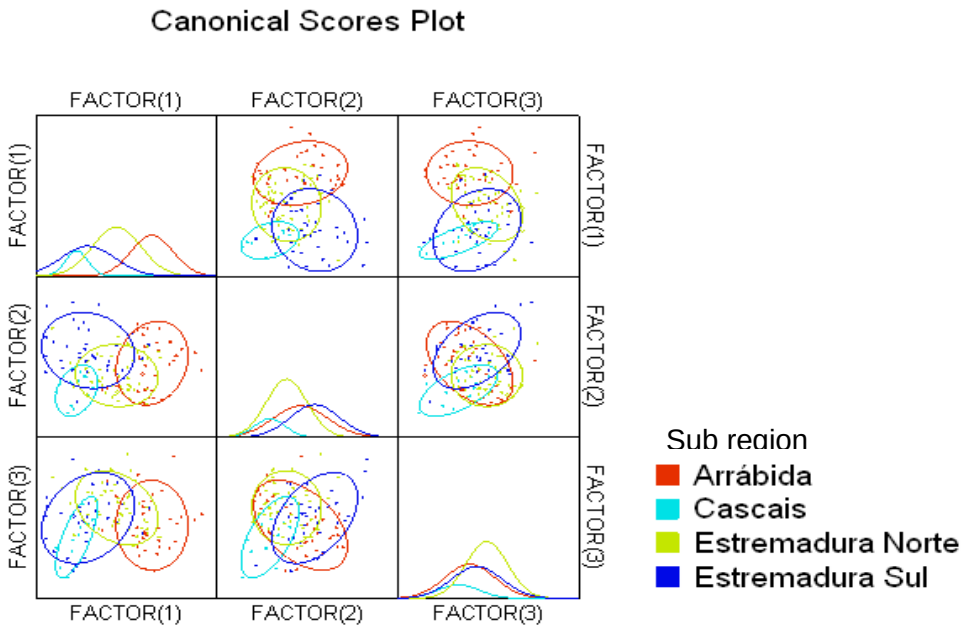

Fig. 6. Canonical variate plots displaying spatial differences in elements signatures in core otoliths of *Lipophrys pholis* embryos from the CW sub region

Jackknife classification overall accuracy was 70% for all CW embryos (Table 4)

Table 4. Jacknife classification matrix of *L. pholis* embryos based on core otolith elements used in LDFA in CW sub regions.

		Predicted Region				
		Estremadura Norte	Estremadura Sul	Cascais	Arrábida	% Correct
Real region	Estremadura Norte	28	8	4	10	56
	Estremadura Sul	5	21	4	4	62
	Cascais	0	1	11	0	92
	Arrábida	8	0	1	27	75
	Total	41	30	20	41	70

Single elemental composition of cores showed no significant differences among the SW sub regions (ANOVA, P > 0.05) for all element/Ca ratios except for Sr (ANOVA, P < 0.05) (Fig. 7).

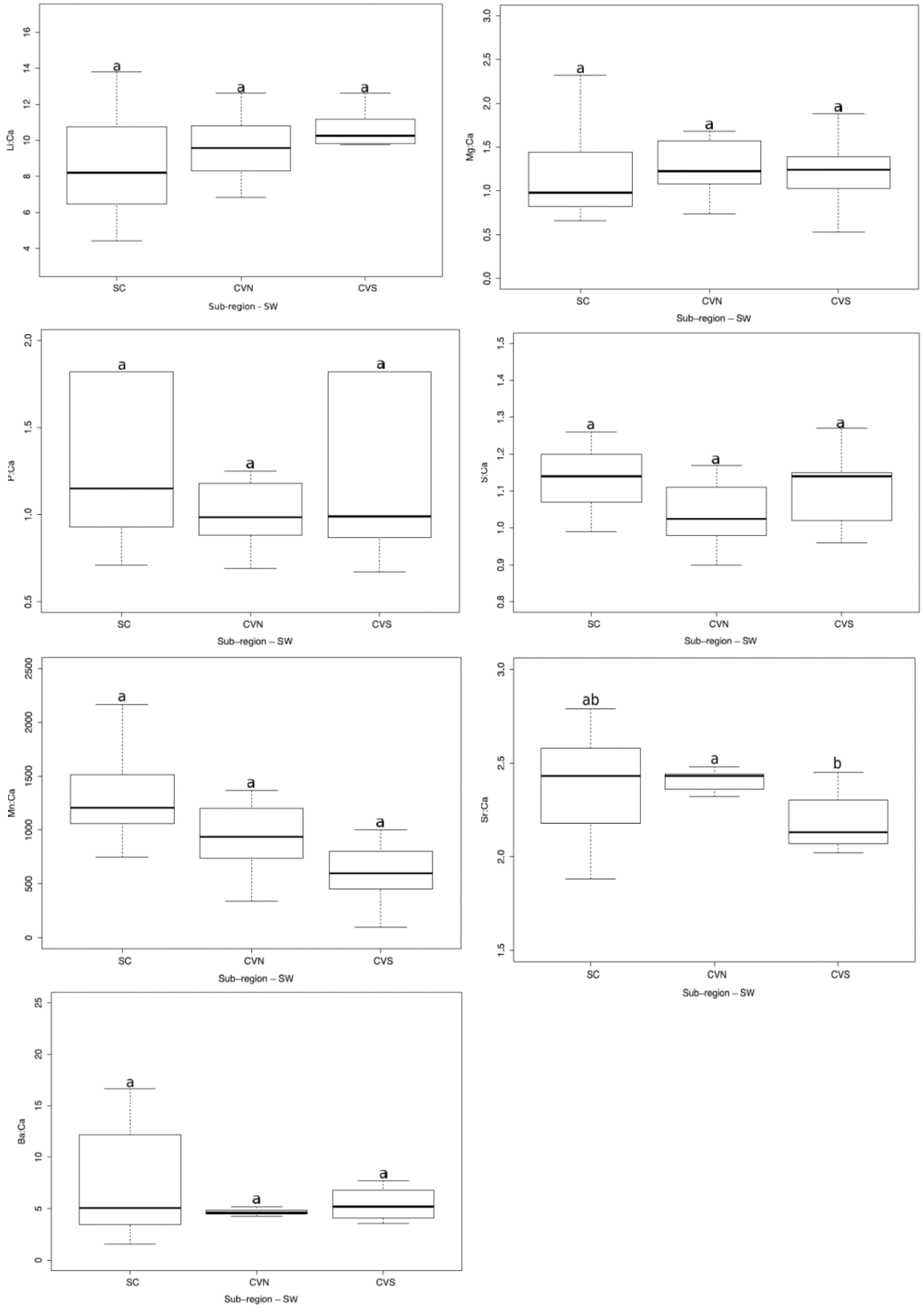

Fig. 7. Molar elemental concentrations (box-plots) from core's otoliths of embryos between SW sub regions. The locations marked with the same letter above the error bars are not significantly different concerning the elemental concentrations (P > 0.05). Ratios are given in µmol element mol^{-1} calcium for Li, Mn and Ba. For Mg, P, S, and Sr ratios are given in mmol element mol^{-1}

For SW region, MANOVA indicated the existence of significance differences in the multi – element signatures of the otolith cores (Pillai trace, $F_{10,70}$=4.087 P<0.05) Furthermore, LFDA showed three distinct groups with some overlap between sub regions (Fig. 8).

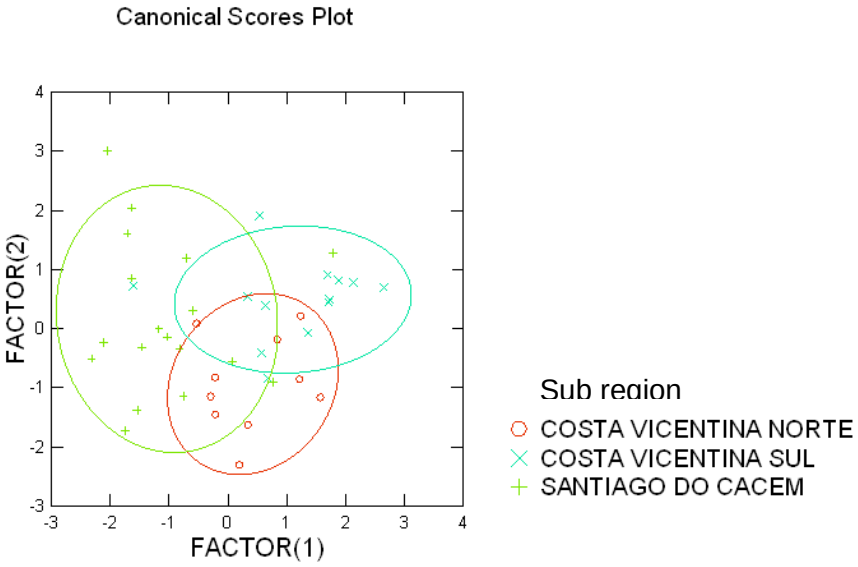

Fig. 8. Canonical variate plots displaying spatial differences in elements signatures in core otoliths of *Lipophrys pholis* embryos from the SW sub region

Jackknifed classification accuracy was relatively high for SW region showing an overall mean of 71% (Table 5).

Table 5. Jacknife classification matrix of *L. pholis* embryos based on core otolith elements used in LDFA in SW sub regions.

		Predicted Region			
		Santiago do Cacém	Costa Vicentina Norte	Costa Vicentina Sul	% Correct
Real region	Santiago do Cacém	14	3	1	78
	Costa Vicentina Norte	1	7	2	70
	Costa Vicentina Sul	3	2	8	62
	Total	18	12	11	71

Establishment of natal origin of juveniles

The contributions of the embryos to the new settlers populations from sub-regions to regions, or sites to sub-regions were presented in Fig. 9.

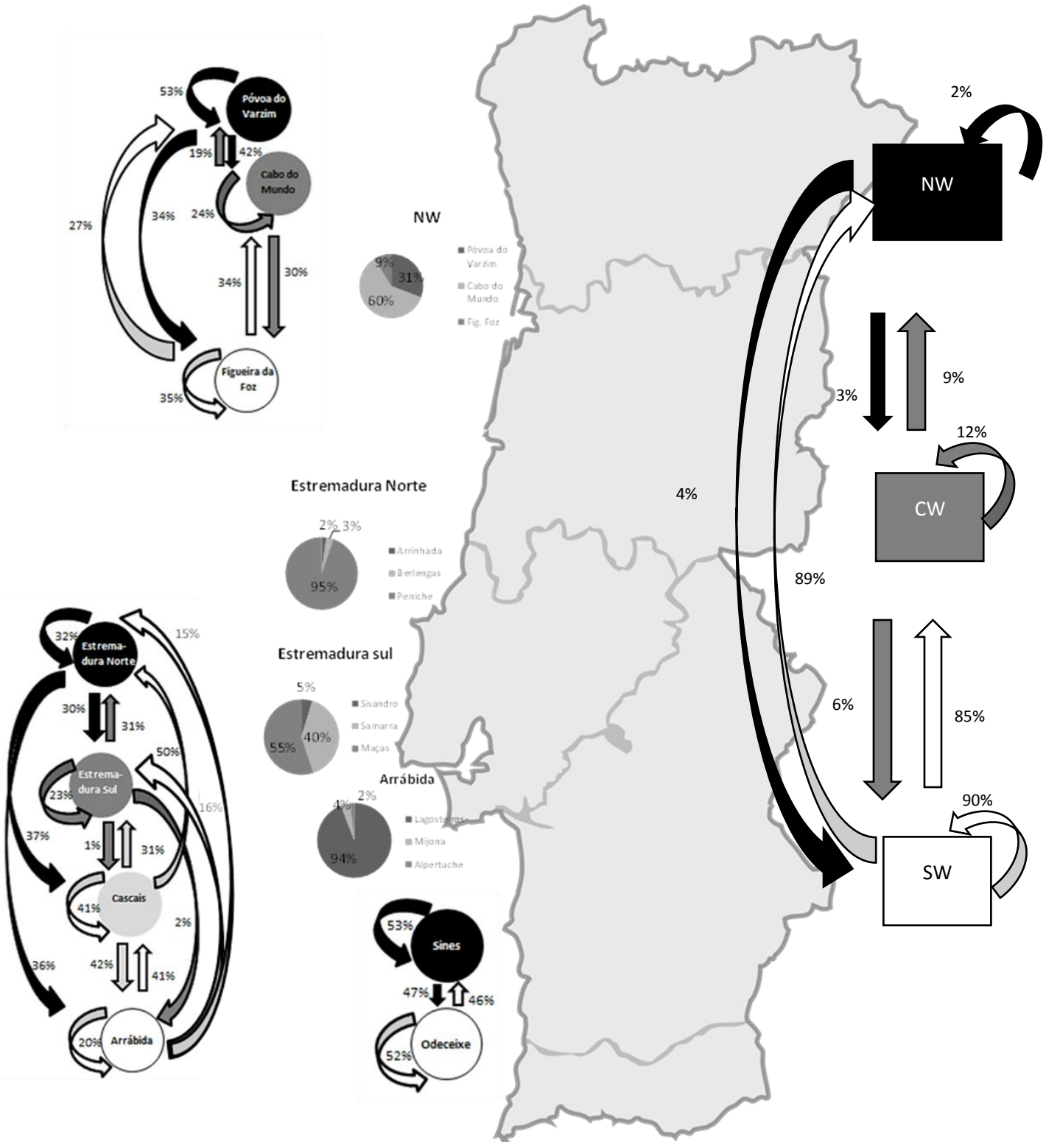

Fig. 9 Predicted dispersal pathways of *Lipophrys pholis recruits* among the 3 main regions along the west coast of Portugal, from mid April- May 2013. Color and numbers above arrows represent the natal origin and percentage, respectively of recruits that originated from the region at the base of the circle.

In NW, Cabo do Mundo showed the greatest contribution as source populations with 60% of the individuals, followed by Póvoa do Varzim with 31% and Figueira da Foz, was the lowest contribution with only 9%.

Recruits collected in Póvoa do Varzim registered a self-recruitment of 53%, Figueira da Foz with 35% and Cabo do Mundo with 24%. Póvoa do Varzim was the site that contribute most in terms of recruits exported to Cabo do Mundo.

For the CW region, Peniche was the site that most contribute as source population for Estremadura Norte sub region; Praia das Maças was the site that contribute most for Estemadura Sul population; and Lagosteiros was the greatest contribution for Arrábida sub region source.

The juveniles collected in Estremadura Norte (32%), Estremadura Sul (23%), Cascais (41%) and Arrábida (20%), were originated from that regions as a self-recruitment mechanism.

Cascais was the sub region with higher self recruitment (41%) and that most contributed in terms of recruits exported for Estremadura Norte (50%), Arrábida (42%) and Estremadura Sul (31%).

SW registered similar values for self-recruitment for both Odeceixe and Sines, with 52% and 53%, respectively. Both sites contribute with the same recruit's percentage between them, with 46% between Odeceixe para Sines and 47% for Odeceixe.

If we considered the overall main regions the SW was the main contributor for all the Portuguese regions ranging between 85% and 89% (Fig. 9).

Discussion

Factors, such as water physico-chemical proprieties (e.g. chemical composition, salinity and temperature) and fish physiology (e.g. age, growth, metabolism and ontogeny) can all potentially influence the deposition of chemical elements into otoliths (Campana et al., 2000). In contrast, incorporation of physiologically important elements, such as P, Cu, K and S, do not seem to be affected by their relative concentration in the environment (Campana, 1999). For Mn:Ca here is no clear evidence of links between variation of these ratios in otoliths and ambient concentrations of Mn and variation appears related to poorly understood endogenous process (Martin & Wuenschel, 2006; Hamer and Jenkins, 2007).

However, changes in otolith Sr:Ca ratios have been considered related to environmental factors such as water temperature (Radtke 1989; Townsend et al. 1992, 1995) and salinity (Secor 1992; Secor et al. 1995; Radtke et al. 1996). Calcium (Ca) can be partially substituted for strontium (Sr) during deposition in otolith, because Sr has the same valence as Ca, as well as a similar ionic radius (Amiel et al. 1973).

In the hereby study there was a significant variation in single and multi-elemental chemistry of *L. pholis* otolith embryos cores among the sub-regions and/or sites within the NW, CW and SW Portuguese sampling regions. Only P, S and Mn uni-elemental concentrations were similar between sub-regions. Furthermore there is a moderate to high accuracy of the percentage of reclassification success to the sampling location of individuals expressed in the jackknifed matrices. It seems that natal chemical signatures are somewhat spatially specific for *L. pholis*. In North sub regions, Sr and Mn were the only elements with different concentrations; in CW sub regions all elements have significant variation between sampling sites; and in SW region, only Sr concentration on otolith embryo were responsible for discriminating sites signature. However multi-elemental signatures are highly different between sub-regions and/or sites.

The main contribution for NW region recruitment process was Cabo do Mundo, with 60% followed by Póvoa do Varzim with 13%.The small contribution of Figueira da Foz's for the NW region recruitment process may be related the oceanography and hydrographic settings of the area. In fact, this site is majorly influenced by Mondego's river estuary, the 5th largest Portuguese river with a freshwater input of 108 m^3 s^{-1}. Furthermore, the Ria de Aveiro, a coastal lagoon located in the northwest Portuguese coast and the Douro River, both located between Figueira da Foz and the remaining two sites are responsible for a freshwater input into the Portuguese inner shelf of 40 m^3 s^{-1} and 488 m^3 s^{-1} respectively (www.maretec.moid.com; Vieira & Bordalo 2000). Such may possibly generate a natural barrier in the NW region, thus preventing the recruitment of Figueira da Foz born individuals into the aforementioned remaining location.

Peniche is the site that contributes most to Estremadura North sub region, once the nearshore wave propagation in this area were significantly disturbed by the complex morphology of the Nazaré canyon head. The NW dominant offshore wave direction clearly benefits the perpendicularly oriented Peniche - Nazaré reache thus becoming the near shore area with higher wave energy resource (Cunha and Gouveia 2016).

Arrábida Bay represent important discontinuities along the central Iberian west coast as they are more sheltered from upwelling pre-valent winds and under direct influence from one of the

major estuaries, Sado, which basin drain in some of the most heavily industrialized areas of Portugal (Caeiro et al. 2005, Costa et al. 2011, Santos-Echeandía et al., 2012).

The hydrodynamics of the Sado estuary is forced by the tide and by Sado River discharge. The mean flow of the Sado River was considered to be 10 m^3 s^{-1} . The contribution of Lagosteiros to the Arrabida Sub region, may be related with the important recirculation inside the estuary right in front of the town of Setúbal which send the born individual upper to north sites (www.maretec.moid.com).

20% to 53 % of early juveniles may be returning to their natal population (self-recruitment), but others came from other areas mainly from southern locations. It also means that fish larvae disperse away from their natal population so that local populations operate as 'open' systems driven by recruitment of larvae from other sub-populations suggestion a metapopulation structure. This pattern has been already reported for coral reef fishes (Jones et al. 1999)

Furthermore, unexpectedly, the SW was the main contributor for the 2013 cohort for all the main regions and larvae are probably driven by the northward flow of the Portuguese Coastal Counter Current (Fıúza, 1984) during the breeding season (winter) (Faria et al., 1996).

This data suggests a long-distance dispersal for *L. pholis* fish populations. However this data should be looked with care and further studies should assess if this pattern persists in the subsequent years.

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CHAPTER 5

Concluding remarks and future perspectives

Concluding remarks and future perspectives

Otolith microstructure can provide useful information on life history traits of fish allowing to determine pelagic larval durations, to reconstruct settlement patterns and to investigate Information provided by annual and daily increments (Campana & Neilson, 1985; Choat & Robertson, 2002; Thorrold & Hare, 2002). Age determination of fishes based on periodic growth increments in otoliths has become a routine tool in fisheries science over the last century. Studies increment width data are related to the growth of individual fish represent more powerful application of otolith microstructure examination (Campana & Neilson, 1985)

Additionally, otoliths structure is particularly useful to reveal ontogenetic or environmental patterns changes experienced by individual fish. Specific variation in the environment can induce a specific mark in the otolith structure which could be useful afterwards as a reference mark to compare individual life history trait responses (Sponaugle & Pinkard, 2004). The biological significance of first mark in otoliths is probably species specific and may correspond to several life history events such as yolk-sac absorption, hatching or first feeding (Campana & Neilson, 1985)

Increments formed before hatching or before exogenous feeding may be different in character to those normally referred to as primary increments. In some species embryonic rings are absent or occluded at hatching, in others they are clear distinct, and it is important to be able to identify them as pre-hatching rings (Geffen, 1983; Dabrowski & Tsukamoto, 1986; Karakiri *et al.,* 1991).

During the late embryonic stage of *Lipophrys pholis*, a few micro-increments (8-10) were visible (through SEM), in the center of the otolith, considered to be formed prior to hatching, i.e before the hatching check. These micro-increments are probably related to ontogenetic developmental events that occur during the fish embryonic stage, such vascularization, eye pigmentation or development of other structures (Geffen, 1983). Similar results were observed in *Perca fluviatis* (Kristensen *et al.*, 2008) *Gobiesox marmoratus* and *Sicyases sanguineus* (Contreras *et al,* 2013).

In *L. pholis* settlers, the hatching check are clearly visible in the sagittal otoliths and it was assumed to be the first distinct micro-increment, which marks the onset of daily growth deposition. However, the first feeding check was not distinguished in sagittae probably because of the short duration of the yolk-sac absorption, since the first exogenous feeding occurs one day after hatching (Faria *et al*., 2002). The micro-increments were deposited on a daily basis. (Chapter 2: Ontogenetic development of the sagittal otoliths of *L. pholis* during embryonic, larval and settlement stages)

Experimental rearing appears to be one of the best methods for studying early growth of larval fishes (Geffen, 1992). The overall otolith daily growth rate (OGR) recorded in our work for *L. pholis* larvae and settlers were 0.99 ± 0.63 µm /day and 2.28 ± 0.25 µm/ day, respectively (Chapter 2: Ontogenetic development of the sagittal otoliths of *L. pholis* during embryonic, larval and settlement stages). These results suggest that OGR was higher in settlers compared to larvae. However, these findings should be analyzed carefully, since larvae were reared under laboratory conditions and were probably under sub-optimal growing conditions which frequently induce fish stress and affect growth rate (Lambert 2003). The OGR are however within the values obtained for *L. pholis* recruits (2.25 ± 0.50 µm/ day to 3.03 ± 0.50 µm /day) captured across the Portuguese coast.

In the peripheral region of the otoliths two settlement marks were identified in the early juveniles. The first, referred to as Ia, was characterized by a sharp decrease in increment width across the settlement mark completed within a few increments; and the second one was a multi-increment transition mark, Ib (Chapter 2: Ontogenetic development of the sagittal otoliths of *L. pholis* during embryonic, larval and settlement stages)

Appropriate validation of the periodicity of otolith increments formation is still essential for a correct interpretation of the otolith microstructure (Geffen, 1992). Thus validation techniques were used to determine deposition rate of micro-increments in otoliths of *L. pholis* juveniles. Marking otoliths with tetracycline or other fluorescent compounds is the best method for validating increment deposition rate in larvae or juvenile of unknown age. These individuals may be captured from the wild, marked and retained for duration of experiments (Geffen 1992). In this study both tetracycline hydrochloride (TC) and alizarin (ARS) were very successfully in inducing a mark in the otoliths of all *L. pholis* recruit exposed and appeared as distinct bright red and yellow rings, respectively, when viewed under UV light (Chapter 2:Validation of otolith daily increment in early juveniles of shanny *L. pholis.*).

It was necessary, however, to use a higher concentration of TC to mark the same number of individuals than ARS (400 mg l^{-1}:100 mg l^{-1}, respectively). The fact that TC chelates with the divalent cations dissolved in sea water could be one plausible reason (Vigliola, 1997).

The mortality rate observed during the chemical exposure itself and subsequent growth period for ARS (40%) was higher than for TC (16%). However size of juvenile fish could be an explanation of the high mortality for ARS in this study, once fish used for ARS were significantly smaller than those used for TC (19·9±2·3mm *v*. 26·1±5·3 mm). Smaller fish are probably more vulnerable to the marking and handling procedure.

The daily growth rate during the experimental period was 1·20±0·37 µmday^{-1} and 1·26±0·41 µmday^{-1} for ARS and TC. Assuming that both marking methods had no effect on

fish and otolith growth, similar values were reported for other perciforms (Shcherbich, 2005; Victor, 2007).

For the juveniles, a significantly very good relationship between the number of micro-increments in otoliths and the true age of individuals during the experimental marking period was observed. Furthermore, the obtained slope of the linear regressions was very close to 1. These data clearly indicate that the primary increments in sagittae were deposited in a daily basis in *L. pholis* and can be used as reliable sources of age information for this species.

In blenniids, daily increment periodicity in otoliths has only been validated for molly miller *Scartella cristata* (L. 1758) (Grabowski, 2002), but it was assumed to be daily in *L. trigloides* (Valenciennes 1836) (Macpherson & Raventos, 2005). Otolith daily increment deposition is, however, well known for other related taxa, such as gobiids (Hernaman *et al.*, 2000).

Several studies have suggested that the pelagic larval duration seem to be a flexible early life history trait in both littoral and demersal fishes (McCormick, 1999; Sponaugle *et al*., 2006; Kendall *et al*., 2013). However studies on the conservative or flexible pattern of the size at settlement in fish are, at present, scarce (Juncker *et al*., 2006).

Recruits collected across the Portuguese coast (Cabo do Mundo, Peniche, Vale do Homem and Olhos de Água) were used to back calculate (through otolith microstructure) the spawning, hatching and settlement dates (identified through the settlement marks) for each fish captured (chapter 3: Pelagic larval duration, size ate settlement and coastal recruitment of *L. pholis*). Similarly, to settlers, in recruits the settlement type Ia and Ib were identified and recorded in 62% and 38% of the individuals, respectively. It is plausible that these settlement marks reflect *L. pholis* individuals that settled successfully (type Ia) or individuals that settled in an unsuitable (or occupied) habitat and then moved before settling again (type Ib).

The estimate of the pelagic larval duration (PLD) from otolith microstructure of *L. pholis* revealed a latitudinal pattern, i.e. a general shortening of the pelagic larval duration from north to south regions: Cabo do Mundo: 73 ± 1 days; Peniche: 64 ± 1 days; Vale do Homem: 57 ± 2 days; Olhos de Água: 58 ±3 days), being the overall PLD was 64.4 ± 0.8 days.

Furthermore, 30% of the variation in PLD was explained by individual mean temperatures experienced by larvae calculated from local sea surface temperature. These results corroborate that temperature is a dominant influence on pelagic larval duration, which decreases exponentially with increasing temperatures across species and populations of marine fish (McCormick & Molony, 1995; Benoît *et al*., 2000; Green & Fisher, 2004).

The recruit's growth history of the *L. pholis* is reflected in the changes of width of the otolith increments. Two different otolith increment width profiles is observed on sagittae. A regular increase of the increment width was observed from the hatch check to the following 35 to 55 days for all individuals/sites. Afterwards there was a decrease of the increment width that

reached the initial value at the otolith edge (110 to 120/130 days) for the northern individuals. For the southern individuals after this initial decrease until the 65 days, there was a steady increase in the increment width through the otolith radius with a final drop in the otolith edge. An abrupt increase of the width of increments took place in the zone where the settlement occurred (40 to 55 days post-hatching). Otolith increments widths recorded in *L. pholis* are consistent with the values reported for other related fish families (Wilson & McCormick, 1999). Moreover, micro-increment widths measured in *L. pholis* otoliths showed a regional variation and sites with higher sea surface temperatures had higher otolith growth rates, which is particularly evident in the peripheral rings.

A consistent size at settlement was found in all sampled sites which emphasizes the fact that fish need to reach a minimum size to begin to settle (~19mm). Longer planktonic periods in northern waters than in southern waters suggest that slow-growing juveniles remain in the plankton until they reach appropriate size, perhaps in response to environmental conditions, namely due to sea water temperature exposure.

The distribution of activities, birth, spawning and settlement over the lunar cycle when estimated for each individual suggested that these biological events were apparently acyclic and continuous over the lunar cycle.

In the Portuguese coastal waters, *L. pholis* breeding season occurs from early autumn to middle spring (early October to late May) and nests containing eggs can be easily observed in the rocky beaches (Faria *et al*., 1996). In the northern parts of its geographical range, *L. pholis* has been studied intensively and the breeding season appears to be shorter and spawning takes place during spring and early summer (March/April to August) (Qasim, 1957). Biological traits such data on age, growth, sex-ratio and sexual developmental stages are unknown for this species in the southern locations, and probably differ from the northern geographic locations.

In our study (Chapter 3: Age, growth and sex of the shanny, *Lipophrys pholis* (Linnaeus, 1758) (Teleoste Blenniidae), from the NW coast of Portugal) marginal increment analysis shown that one translucent and one opaque zones were formed each year in the sagittal otoliths. Age for the *L. pholis* ranged from 0 to 6 years and similar results were found in the British islands once individuals does not reach usually more than six years of age (Qasim, 1957; Bowers, 1960; Dunne, 1977). In our study individuals from both sexes less than one year with total length around 70 mm are also already mature. The hereby data is according with other authors that reported that both sexes of *L. pholis* could be mature around one year old with 80 mm length in the Portuguese coast (Faria *et al.,* 1996; Monteiro *et al.,* 2005). In northerly populations with colder water maturation is achieved after a period of two years (Milton, 1983). The VBGC estimates from the otoliths gave the result of Linf = 153 mm, k =

0.38 mmyear^{-1} and t0 = -1.16 and Linf = 176 mm, k= 0.30 mmyear^{-1} and t0= - 1.24 for females and males, respectively, However there are no statistical differences (Linf, k and t0) between females and males (P > 0.05), which agree for other small blenny fish: Linf = 102 mm and t0=-1.62, or Linf = 97 mm and t0 = -0.77 for females and males of *Omobranchus punctatu* respectively (Ismail & Clayton, 1990).

The maximum GSI for males and females coincided with the breeding season (November and March) and lower observed GSI values were recorded in June, which falls also within the values by others authors (Ferreira *et al*., 2011 and 2012).

Furthermore, some stages of sexual development occur during the same season in males and females, and several germinal cells are observed also at the same time within a single ovary or testis indicating that *L. pholis* is an asynchronous and multiple spawner. The annual variation of the fish condition and hepatossomatic indexes appears to be related with the mobilization of the somatic reserves prior to reproduction.

Information available about the mitochondrial DNA study of *L. pholis* found no significant population genetic structure along the Portuguese coast and attribute the genetic homogeneity of this species to an efficient gene flow as result of the oceanic dispersal of the planktonic larvae (Azeiteiro *et al.*, 2006). Meanwhile more information about the movement patterns, population structure and habitat connectivity is at present scarce.

Otolith stable isotope ratios have been used to reconstruct environmental temperatures, differentiate among groups, infer metabolic history and reconstruct migration patterns of fish (Campana, 1999). In our study, the isotopic composition of the whole otoliths of *L. pholis* juveniles was assessed at small (sites) and large (regions) spatial scales ranging from three to hundreds of kilometers, respectively. The source of stable isotopes incorporated into the otolith varies according to the element. Oxygen appears to be incorporated into the otolith with isotopic ratios which are nearly identical to those of the ambient water, although they are also influenced by temperature and salinity (Campana, 1999). Our results suggest that both seawater temperature and salinity caused differences in otolith $\delta^{18}O$ among the four main sampling regions.

The inter-regional variation in $\delta^{13}C$ was most likely related to slight differences in the diet, growth and/or metabolism of fish. The distinct isotopic signatures, at least for the northern and central regions, suggested low levels of connectivity across large spatial scales during the juvenile stage. Furthermore, similar isotopic signatures within the same region indicated some degree of larval oceanic retention at short-spatial scales. (Chapter 4: Movement and connectivity in the fish *Lipophrys pholis* (Linnaeus, 1758)) revaled by otolith oxygen and carbon stable isotopes).

In the study with use of otolith elemental signatures (Chapter 4: Use of elemental signatures as natural tags to evaluate the larval dispersion, coastal recruitment habitat connectivity and population structure of *Lipophrys pholis),* laser ablation inductively coupled plasma mass spectrometry was used to measure the concentration of 7 informative elemental ratios in the otolith's core. Molar ratios of Li/Ca, Mg/Ca,P/Ca, S/Ca, Mn/Ca, Sr/Ca and Ba/Ca show that natal chemical signatures are somewhat spatially specific for *L. pholis*. However multi-elemental signatures are highly different between sub-regions and/or sites. The population connectivity matrix identified diferente dispersal pathways for *L.pholis* embryos. Cabo do Mundo was an important source population for NW region. For the CW region, Peniche was the site that most contribute as source population for Estremadura Norte sub region; Praia das Maças was the site that contribute most for Estemadura Sul population; and Alpertuche was the greatest contribution for Arrábida sub region source. SW registered similar values for self-recruitment for both Odeceixe and Sines sites. 20% to 53 % of early juveniles may be returning to their natal population (self-recruitment), but others came from other areas mainly from southern locations. It also means that fish larvae disperse away from their natal population so that local populations operate as 'open' systems driven by recruitment of larvae from other sub-populations suggestion a metapopulation structure. Furthermore unexpectedly the SW is the main contributor for all the main regions and larvae are probably driven by the northward flow of the Portuguese Coastal Counter Current during winter suggesting that long-distance dispersal is the norm for *L. pholis* fish populations. However this data should be look with careful and further studies should assess if this pattern persist in the following years.

Future research must include further sampling of embryos and settlers of the same cohort (2014 – analyses in progress) to evaluate the temporal stability of natal otolith geochemical signatures and to assess if the costal recruitment pattern persist within two consecutive years

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